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(54) Modified cells having resistance to retroviral infection

Modifizierte Zellen mit Resistenz gegen Retroviralinfektionen

Cellules modifiées résistantes aux infections rétrovirales

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- **Cell, Vol. 58, No. 1, 14 July 1989, pages 215-223, Cambridge, MA, US; M. GREEN et al.: "Mutational Analysis of HIV-1 Tat Minimal Domain Peptides: Identification of Trans-Dominant Mutants That Suppress HIV-LTR-Driven Gene Expression"**

Description

[0001] The present invention relates generally to the inhibition of retroviral replication by hybridization interference in a host cell system. More particularly, the invention provides cells modified by having resistance to infection by a retrovirus, said modification comprising that said cell contains a vector comprising a polynucleotide which is transcribed to RNA within said cell; the progeny of such cells as well as cells and/or the progeny thereof as a medicament, for example conferring retroviral resistance to retroviral infection upon cells.

[0002] Retroviruses are the causative agents for an increasing number of diseases of higher organisms including; AIDS, HIV, various leukemias, feline leukemia, murine leukemia, several avian leukemias, various sarcomas of mice, rats, monkeys, birds, and cats, and other lymphotrophic diseases of man, including Adult T-Cell leukemia. Acquired Immune Deficiency Syndrome (AIDS), the recently most noteworthy of these diseases, is caused by a retrovirus which has been called HTLV-III, LAV, RAV or most recently HIV. Coffin et al, *Science*, 232:697 (1986). HIV is one of a group of retroviral diseases which attacks the T4 lymphocytes thereby destroying the body's immune system. Anderson, *Science*, 226:401-409 (1984); Weiss, *In RNA Tumor Viruses-II*, vol. 2, Cold Spring Harbor Laboratory, pp. 405-485 (1985). The disease is uniformly fatal and no cure has been developed which either kills the virus *in situ* or replaces the lost elements of the body's immune system. Some experimental drugs such as HPA-23, azidothymidine and suramin show limited effects in stopping the virus, and immunomodulators such as thymostimulin and isoprinosine hopefully will bolster the patient's malfunctioning immune system, but to date there is no proven therapy or cure for the AIDS patient. It is also unlikely that a traditional vaccine for the virus will be developed for quite some time due to the wide variation in antigenicity of various strains of the virus.

[0003] Retroviral diseases differ from many other viral diseases in that the infective agent, a retrovirus, eventually becomes integrated in the host cell's genome. The retrovirus inserts its genome into a host chromosome, such that its genetic material becomes part of the genetic makeup of the infected cell, and is then replicated with the cell as the cell divides and multiplies. It is this characteristic which makes retroviruses especially persistent and immune to traditional anti-viral treatment. There is as yet no way to kill the retrovirus without killing the host cell. Thus, there is no proven cure, nor is there any proven effective vaccine or pharmacological agent against any retroviral disease.

[0004] Details of the life cycle and replication of retroviruses are discussed at length in Weiss et al, *RNA Tumor Viruses*, vols. 1 and 2 (Cold Springs Harbor Laboratory 1984). Fig. 1(B) summarizes a model of a retrovirus life cycle. The life cycle of retroviruses is unique among viruses. The cycle begins when an infectious

particle enters a host cell and releases two identical RNA molecules. These molecules are "reverse transcribed" by special viral enzymes to produce double-stranded DNA which circularizes and inserts into the host chromosome. Fig. 1(A) summarizes a model of the synthesis of double-strand DNA from viral RNA. The inserted DNA virus or "pro-virus" is structurally very similar to a normal host gene. It is transcribed to produce RNA, like any host gene. This RNA can then be processed in three ways: a) it can be directly translated into certain viral proteins, b) it can be processed and spliced, and then translated to produce other viral proteins, or c) it can be packaged, along with various viral proteins to make a newly infectious particle. In the case of HIV, the infectious particles continuously "bud off" the infected cells and bind to new uninfected cells, beginning the cycle over again.

[0005] The retroviral particle which is the infectious agent contains in its interior two single-stranded positive-sense viral RNA molecules each between 7,000 to 11,000 nucleotide bases in length. These viral RNA's combine with certain viral proteins to form a viral core; the core being surrounded by a membrane. Inbedded in the membrane are viral glycoproteins which can specifically bind the viral particles to the appropriate host cell system. The viral core is assembled within the host cell and exits from the host cell, taking some of the host's membrane with it. Hence the membrane of the viral particle is derived directly from the host cell. The particle travels to a new uninfected host cell, and due to the glycoprotein on its exterior binds to the new host cell and the life cycle repeats. Once the virus enters the cell, it is disassembled, releasing the two identical viral RNA molecules. These molecules are each composed of a sequence having specific functional regions making up the viruses "genomic structure".

[0006] The genome of any retrovirus is divided into three regions: the 5' terminus, the 3' terminus and a central region containing genes coding for proteins. The 5' terminus is further divided into four functional regions: the terminal redundancy (R), a unique sequence (U5), the primer binding site (PB- or PBS) and an untranslated sequence (L). The L region may contain a splice donor site for subgenomic mRNA. The 3' terminus is further divided into three functional regions: the primer-binding site for positive strand DNA synthesis (PB+ or PBS), a unique sequence (U3) and another copy of the terminal redundancy (R). The U5, U3 and R regions are sometimes collectively referred to as the Long Terminal Repeat (LTR) region. Components of the LTR region are involved in integration of the retroviral genome into the genome of its host. All retroviruses contain these highly conserved regions. These regions are further described by Weiss et al, *supra*, pp. 262-296.

[0007] The production of DNA from the infectious RNA occurs by a complex process called reverse transcription. The viral reverse transcriptase enzyme first complexes with a specific tRNA molecule supplied by

the host cell. For example, in the case of the AIDS-related virus, it is lysine tRNA which complexes with the reverse transcriptase. The 3' end of the tRNA molecule remains free to hybridize with the primer binding site (PBS) of the retroviral genome. This is a sequence within the virus, which is complementary to the 5' end of the tRNA. Once the virus/enzyme/tRNA complex has been formed, the enzyme can make a new DNA molecule, using the RNA virus as a template, and using the tRNA as a "primer". As the process proceeds, the RNA of the resulting RNA/DNA complex is degraded, leaving single-stranded DNA. This process begins internally at the PBS site and proceeds to the 5' end of the RNA virus, where the process is stalled and regresses slightly, leaving a single-strand DNA "sticky end". At this point the enzyme/DNA complex has to "jump" to a new template at the 3' end of the virus. This jump, termed the first jump, is possible because the newly synthesized DNA is complementary to the other R region at the 3' end of the virus. After this jump, reverse transcription continues around to the point of the primer binding site.

[0008] After the "first jump" and while reverse transcription continues, second-strand DNA synthesis begins from the poly-purine site upstream of the U3 region. This DNA second-strand synthesis continues in the opposite direction from the first-strand DNA synthesis and proceeds through the primer binding site. The RNA primer molecule is consequently degraded, leaving a short residual region of second-strand DNA extending from the region of double-strand DNA. At this point the enzyme/DNA complex needs to make a "second jump" to a new template, this time jumping to the "sticky end" of the second strand DNA. This is possible because of complementation between the first and second strand DNA molecules in the region of the primer binding site. After hybridization of the complementary ends, reverse transcription can continue using the second-strand DNA as a template. This subsequently results in displacement of the first strand DNA, past the site of the first jump, up to the point where the second-strand synthesis begins. Second-strand synthesis which was stalled at the PBS site prior to the second jump, can also continue after this jump, and proceeds to the 5' end of the first-strand DNA. The result of this process is a double-stranded DNA molecule with additional redundancies at both ends. Note that the DNA genomic structure differs from the RNA genomic structure in having a redundant U3 region added to the 5' end, and a redundant U5 region added to the 3' end. This occurs because the reverse translation process copies more than one full length of the RNA genome. Note also that this genomic structure now resembles a normal gene, with U3 being the promoter, with structural genes in the center, and a U5 tail.

[0009] The exact process of how the DNA virus inserts into host chromosomes is not known. It is known that the DNA virus first becomes a circle, and that this involves the short inverted repeat sequences at the

ends of the virus. These inverted repeats may be involved in some form of DNA hybridization which brings the ends of the virus together, allowing circularization. Subsequently, insertion into the chromosome is generally assumed to be mediated by an enzyme which recognizes the splice site in the circle and directs insertion of a single copy of the virus into a random site within the host chromosome.

[0010] The transcription of viral DNA from the DNA pro-virus within a chromosome occurs in a manner similar to the transcription of any host gene. The U3 region functions as a polymerase II promoter and transcription begins at the beginning of the R region. The U3 promoter like eukaryotic promoters generally requires a transcriptional activator protein, which turns the promoter "on". Transcription proceeds through most of the pro-virus and is terminated at the end of the 3' R region. As a result, the transcript is a recreation of the smaller and infectious single-strand RNA genome. A poly-A tail is attached to the 3' end of this RNA and the 5' end is capped, making this molecule similar to normal host messenger RNA.

[0011] The RNA which is transcribed from DNA can be directly translated into protein, like any mRNA within the host. The GAG and Pol proteins are produced in this way and are subsequently cleaved into several smaller proteins involved in viral assembly and reproduction. In such a case, the 5' end of the RNA binds to a ribosome and protein translation begins at the first AUG codon initiation triplet of the coding sequence closest to the 5' end of the RNA molecule. Translation is terminated by one of the standard "stop" codons. Genes which are distant from the 5' end of the viral RNA cannot be directly translated because of the intervening genes, such as GAG. Such intervening genes can be removed by a splicing process which involves breaks at specific sites in the RNA molecule, and re-ligation of the appropriate pieces. In this case, the 5' end of the RNA molecule is unchanged, and binds to the ribosome as before, but now the first AUG codon where translation begins is not at the beginning of the GAG sequence, but at the beginning of some other coding sequence further downstream.

[0012] Some viral RNA is not translated into protein, but is packaged into infectious viral particles. Such packaging involves the binding of certain viral proteins to specific sequences of the viral genome. For example, in the RSV viral system, it is part of the GAG sequence which is one of the parts of the genome which binds to and is recognized by such proteins and have been shown to be necessary for packaging of the RNA. The RNA which is packaged into viral particles does not appear to be reverse-transcription-competent until "maturation" of the particle, i.e., after it has existed away from the host cell.

[0013] All retroviruses, including HIV, once inserted into the host chromosome, must have their genes translated into viral proteins. If viral proteins are not abun-

dant, the retrovirus cannot efficiently propagate to other cells and is not cytopathic to the infected host cell. Dayton et al, *Cell*, 44:941-947 (1986); Fisher et al, *Nature*, 320: 367-371 (1986). Such proteins are not produced without the proper functioning of certain viral regulatory proteins. One of the key DNA/RNA-binding regulatory proteins for the retrovirus HIV is the TAT protein. Keegan et al, *Science* 231: 699-704 (1986). The TAT protein is essential to protein translation of HIV, and possibly also involved in RNA transcription. It is apparent that the TAT protein recognizes and binds to the nucleic acid sequence corresponding to the 5' end of the R region. A second activator gene ART has also been shown to be important in HIV translation. Sodroski et al, *Nature*, 321: 412-417 (1986). DNA/RNA binding of the previously described activator proteins is essential to HIV replication. Therefore, introducing genes into host cells, i.e., gene therapy for humans or germline transformation for animals, which will code for modified proteins of the retrovirus which compete or interfere with TAT or ART, will effectively block retrovirus replication.

[0014] Past research efforts have been predominantly confined to two traditional anti-retroviral approaches: immunological prevention and pharmacological therapy. Unfortunately, neither of these approaches appears to be very promising for control of retrovirus diseases. At best, an effective vaccination might reduce risk of infection in healthy individuals, but it would not be expected to cure an infected individual. Also, chemical repression of virus diseases has not generally been effective in eradicating any persistent virus, and certainly would not be expected to eradicate a retrovirus. Anti-viral chemicals tend to slow the progress of a virus and to bolster native defense mechanisms, but chemical treatments must be continuously applied and typically have undesirable side effects.

[0015] For these reasons, it is doubtful that any retrovirus disease can be cured by the traditional anti-viral approaches. An alternative approach to inhibiting retrovirus replication is genetic inhibition by introducing gene constructs into host cells, i.e., gene therapy or germline transformation, which will confer cellular resistance by hybridization interference.

[0016] The inhibition or modulation of the various steps in the retroviral replication process by DNA or RNA which will hybridize and block viral sequences has been termed "hybridization interference". Green et al, *ANN. REV. Biochem.*, 55:569-97 (1987). There are essential steps in retrovirus replication which require nucleic acid hybridization. Gilboa et al, *Cell*, 16: 93-100 (1979). If any of these replication steps are blocked by pre-binding of the essential sites in the retrovirus genome; or binding of proteins or other cellular constituents in the retrovirus genome, to molecules coded for by genetically engineered nucleic acid sequences in the host cell the retrovirus replication process can not proceed. Note, that "Hybridization Interference" has also been referred to as an "Anti-sense approach". Green et

al, *ANN. REV. Biochem.*, 55:569-97 (1987). However, an ambiguity exists in that "sense" and "anti-sense" only apply to sequences coding for proteins, and gene constructs are disclosed herein which target retrovirus sequences not coding for proteins. Consequently, as used throughout the specification and appended claims, "Hybridization Interference" or "Anti-sense RNA" should refer to the use of RNA or DNA to bind with nucleic acid, protein or other cellular constituents to inhibit retrovirus replication.

[0017] The effectiveness of the anti-sense RNA approach has been demonstrated in several model viral systems. It was demonstrated in the SP bacterial phage system that certain messenger-RNA-interfering complementary RNA (micRNA) can have very significant anti-viral effects, as seen by reduced plaque number and plaque size. Coleman et al, *Nature*, 315: 601-603 (1985).

[0018] In addition, it has been suggested that the replication and cell transformation of the Rous Sarcoma Virus (RSV) was inhibited by a specific synthetic tridecamer oligodeoxynucleotide. Zamecnik and Stephenson, *Proc. Natl. Acad. Sci.*, 75: pp. 280-288 (1978). The synthetic complementary tridecamer was introduced extracellularly into the cytoplasm of chick embryo fibroblast cells infected with RSV virus, thereby blocking RSV replication by hybridization competition. However, the tridecamer was not incorporated into the host genome or any other genetic vehicle, such that neither the sequence, nor an equivalent coding sequence, would replicate in the cell. This is a chemotherapeutic approach to inhibiting virus replication, and not gene therapy.

[0019] Another publication has shown that synthetic exogenous oligodeoxynucleotides complementary to regions of the HIV genome inhibit virus replication and gene expression in cultured cells. Sequences of exogenous synthetic oligodeoxynucleotides 12, 20, and 26 nucleotides in length were tested on infected cells. Zamecnik et al, *Proc. Natl. Acad. Sci.*, 83:4143-4146 (1986). Again, the oligodeoxynucleotides are exogenous and were not incorporated into the host genome or another vehicle which would provide for the replication or maintenance of the tridecamer.

[0020] Finally, the anti-sense RNA-mediated inhibition on the replication of avian retrovirus in cultured cells was suggested using natural gene sequences derived from the neomycin resistant gene of the bacterial transposable element Tn5. To et al, *Molecular and Cellular Biology*, vol. 6, No. 12, pp. 4758-4762 (1986).

[0021] In the field of human medicine, altering the genotype of the host has not been a desirable method of fighting infectious disease. However, it is now believed that gene therapy will be possible in the relative future. Anderson, *Science*, 226:401-409 (1984). As a result, application of the anti-sense RNA approach within the field of medicine may be possible. Presently available gene therapy techniques are only effective for the genetic modification of bone marrow and blood cells.

Because of this limitation the projected use of gene therapy has generally been assumed limited to the correction of rare hereditary gene defects where such defects center in bone marrow or blood cells. Despite these limitations there are certain pathogens of the blood for which conventional defenses appear inadequate, and where the use of anti-sense RNA inhibition might be feasible. Many of the cells that are infected by retroviruses are derived from hematopoietic stem cells. If these stem cells can be altered by the incorporation of genes which will synthesize RNA molecules that are antagonistic to virus propagation, an efficient method to both effectively prevent and to treat these retrovirus diseases will be apparent. Further, if the expression of the RNA inhibiting genes can be regulated in the desired cells, it has application to other genetic diseases.

[0022] The document WO 87/03451 described a method for conferring resistance to retroviral infection by transforming cells susceptible to such retroviral infection with a polynucleotide directing transcription of RNA that is complementary to a region of the retroviral genome and that is efficacious to interrupt retroviral replication by substantially preventing formation of pathogenic virions in the treated cell and its progeny.

[0023] The document WO 87/07300 relates to the inhibition of HTLV-III by the administration of an oligonucleotide complementary to highly conserved regions of the HTLV-III genome necessary for the HTLV-III replication and/or gene expression.

[0024] The publication "Proc. Natl. Acad. Sci. USA 83 (1086), 4143" discloses possibilities of using oligodeoxyribonucleotides complementary to viral RNA or proviral DNA to inhibit the replication of HTLV-III.

[0025] The document WO 86/05516 relates to a method for conferring resistance to a parasite to a host, wherein a gene fragment is isolated from the parasite, and said fragment or a DNA or RNA segment substantially homogeneous to the gene fragment or to a DNA or RNA sequence functionally equivalent to said gene fragment is inserted into a host.

[0026] It would therefore be desirable to provide methods and compositions for producing RNA which is complementary to an essential retroviral hybridization site within the retrovirus genome selected from the group consisting of the LTR region, the U5 region, the U3 region, the 3'R region, and the AUG start codon region, which would be effective to inhibit retroviral replication.

[0027] Another objective is to provide methods and compositions for expression of a synthetic double-strand DNA fragment coding for an RNA fragment complementary to an essential retroviral hybridization site within the retrovirus genome. It was a further object to provide RNA molecules conferring resistance to retrovirus infection, which are transcribed within a host cell from a nucleic acid sequence introduced into said host cell by transformation with a vector. It was a still further object to provide nucleic acid constructs conferring re-

sistance to retrovirus infection. It was a still further object of the invention to provide cells and progenies of such cells which were transformed with a nucleic acid construct which confers resistance to retrovirus infection to said cells or progenies, respectively. It was a final object to provide said cells and/or progenies thereof as a medicament, in particular as a medicament conferring resistance to retrovirus infections to cells.

[0028] The invention relates to a cell modified by having resistance to infection by a retrovirus, said modification comprising that said cell contains a vector comprising a polynucleotide which is transcribed to RNA within said cell, said RNA being selected from the group consisting of RNA complementary to a nucleic acid sequence within at least one region within the genome of said retrovirus, wherein said region is selected from the group consisting of the LTR region, the U5 region, the U3 region, the 3'R region, and the AUG start codon region, thereby inhibiting, in the infection process, at least one process selected from the group consisting of retroviral replication, reverse transcription, and translation.

[0029] The invention further relates to the progeny of cells as defined above containing the vector defined above.

[0030] The invention also relates to cells and/or the progeny thereof, as above defined, as a medicament.

[0031] The invention also relates to cells and/or the progeny thereof, as above defined, as a medicament conferring resistance to retroviral infection upon cells.

[0032] Cells upon which resistance to infection is to be conferred are transformed with a polynucleotide via a vector. "Transformation" or "transformed", as those terms are used throughout this specification and the appended claims, is intended to cover any and all methods of introducing a polynucleotide and its other attendant nucleic acid sequences, if any, into a cell. Those terms are not intended to be limited to the process of "true" transformation which is known to those in the art. Methods included within those terms include without limitation transformation, transfection, microinjection, CaPO₄ precipitation, electroporation, targeted liposomes, particle-gun bombardment and electro-fusion.

[0033] The polynucleotide used to transform the cell upon which resistance is conferred can be either single- or double-stranded RNA or DNA. The polynucleotide "directs" transcription of a specific RNA molecule in the cell. A polynucleotide can "direct" such transcription by being directly transcribed (e.g., double-stranded DNA in a plasmid) or by coding for nucleic acid which is later transcribed to produce the RNA molecule (i.e., serves as a template for RNA or DNA which is either transcribed or serves as a further template for nucleic acid which is transcribed; e.g., single-stranded RNA in a virus which is transcribed to produce DNA which is incorporated into the host cell genome and in turn transcribed). In addition to the sequence specifically directing the transcription of the operative RNA, the polynucleotide can include a promoter and/or a terminator that will regulate the tran-

scription of the polynucleotide. The polynucleotide may be derived from a naturally-occurring sequence or synthesized *in vitro*.

[0034] The RNA directed by the polynucleotide is complementary or homologous to "one or more" of certain regions within the retroviral genome. In other words, the RNA may overlap between several regions or portions of regions; or the polynucleotide can direct transcription of RNA at several different sites.

[0035] The RNA directed by the polynucleotide must be effective to inhibit the replication of the retrovirus. Inhibition can be exhibited by any decrease in the extent or rate of insertion and proliferation of the retrovirus. Replication need not be completely stopped to constitute "inhibition."

[0036] The polynucleotide is transformed via a vector. Any known vectors, including without limitation viral vectors, retroviral vectors and plasmids, may be used. Preferably the vector is a plasmid. The vector can include a promoter and/or a terminator for regulation of the polynucleotide.

In a preferred embodiment, the vector comprises a first promoter which controls transcription of said RNA within a cell and/or a first terminator which controls termination of transcription of said RNA within a cell. The final construct (vector and polynucleotide) can include one or more promoters and/or terminators including those made part of the polynucleotide as described above. In another preferred embodiment, there is comprised a second promoter which controls transcription of said RNA within a cell, which can, more preferably, be RNA Polymerase III promoter. According to another preferred embodiment, there is comprised a second terminator which controls termination of transcription of said RNA within a cell which can, more preferably, be a RNA Polymerase terminator sequence. The vector can preferably also include a selectable marker for detection and isolation of successfully transformed cells including without limitation antibiotic resistance to neomycin, ampicillin, or xanthine.

[0037] The present invention is applicable to any retrovirus, including without limitation a human T-cell lymphotrophic virus, a human immunodeficiency virus, a lymphadenopathic virus, a leukemia virus, a sarcoma virus, and a virus causing a lymphotropic disease. Such viruses include without limitation HIV, feline leukemia virus ("FeLV"), HTLV-1, HTLV-2, murine leukemia virus and avian leukemia virus. Preferably the retrovirus is HIV, HTLV-1 or FeLV.

[0038] Resistance to retroviral infection is conferred to host cells by hybridization interference, or by modified viral proteins. "Hybridization" is the coming together of single-stranded nucleic acid chains with their complementary nucleotide sequences into double-stranded nucleic acid chains when subjected to hybridizing conditions. "Hybridization Interference" is the inhibition of viral replication by "hybridization" of interfering nucleic acid sequences.

[0039] Cells upon which resistance to infection has been conferred by the above-described methods and their progeny are also disclosed. The Drogeny of the originally transformed cells "contain a sequence which

5 is descendant from" the polynucleotide previously described. A sequence is "descendant" if its history can be traced back to the polynucleotide. The descendant sequence does not have to be an exact copy of the polynucleotide; it need only maintain the function of the polynucleotide in the inhibition process. In essence, a "descendant sequence" must be "homologous" (as defined above) to the polynucleotide. The descendant sequence can have been deleted, inserted, mutated, inverted or altered by other means as long as its functional

15 identity with the polynucleotide is maintained.

[0040] RNA molecules directed by the polynucleotide are also disclosed. Such molecules are (a) being complementary or homologous to a nucleic acid sequence within the genome of a retrovirus, and (b) being effective

20 to inhibit replication of the retrovirus.

In a preferred embodiment, said RNA sequence is complementary to a nucleic acid sequence within one or more regions within the genome of said retrovirus which are selected from the group consisting of the LTR region,

25 the U5 region, the U3 region, the 3'R region, and the AUG start codon region. In a more preferred embodiment, the nucleic acid sequence is a synthetic nucleic acid sequence. It is furthermore preferred that said nucleic acid sequence further comprises a polynucleotide directing transcription of RNA sequence, said sequence redirecting reverse transcriptase enzyme in a nonfunctional transcription direction. More preferred, said RNA sequence is a False Template Sequence or alternatively a False Primer Sequence.

30 35 As described above, the invention also comprises a cell and/or any progeny of such a cell which has been described above as a medicament conferring resistance to retrovirus infection to cells.

It is preferred that said medicament confers resistance to retrovirus infections to cells. However, the invention is not limited to this embodiment.

In a more preferred embodiment, the invention is directed to a cell or any progeny thereof as a medicament conferring resistance to infections of cells by retroviruses

45 selected from the group consisting of human T-cell lymphotrophic virus, human immunodeficiency virus, lymphadenopathic virus, leukemia virus, sarcoma virus and virus causing lymphotropic disease. Particularly preferred are a cell or any progeny thereof as described above as a medicament conferring resistance to HIV infection of cells.

50 It has surprisingly turned out that according to the present invention the cells which have been transformed with a nucleic acid construct conferring resistance to retrovirus infection, in particular HIV virus infection, to said cell, whereby said construct comprises a polynucleotide which when introduced into said cell by transformation directs transcription within said cell of RNA, are particularly useful as a medicament conferring

resistance to HIV infections of cells.

[0041] Fig. 1A and 1B are a schematic representation of double strand DNA synthesis and a general overview of a retrovirus life cycle.

[0042] Fig. 2 is the LTR gene structure for the HIV genome.

[0043] Fig. 3 is the LTR gene structure for the HTLV-I genome.

[0044] Fig. 4 is the LTR gene structure for the FeLV genome.

[0045] Fig. 5 is Table 1 which lists sequences targeted against HIV which are exemplary of polynucleotides employed in practicing the present invention.

[0046] Fig. 6 is the anti-R gene structure for the retrovirus HTLV-I.

[0047] Fig. 7 is the anti-R gene structure for the retrovirus FeLV.

[0048] Fig. 8 is the anti-R gene structure for the retrovirus HIV.

[0049] Fig. 9 is the anti-PBS gene structure for the retrovirus FeLV.

[0050] Fig. 10 is the anti-PBS gene structure for the retrovirus HTLV-I.

[0051] Fig. 11 is the anti-PBS gene structure for the retrovirus HIV.

[0052] Fig. 12 is the anti-AUG and anti-S.D. gene structure for the retrovirus FeLV.

[0053] Fig. 13 is the anti-AUG and anti-S.D. gene structure for the retrovirus HTLV-I.

[0054] Fig. 14. is the anti-AUG, and anti-S.D. gene structure for the retrovirus HIV.

[0055] Fig. 15 is a schematic representation of Complex Gene Constructs 1 and 2 illustrating the Anti-sense polynucleotide structures of the present invention for insertion in either a BamHI or HindIII restriction site, respectively.

[0056] Fig. 16 is the construction of recombinant plasmid HindIII: pRSVneo in the same transcription orientation relative to the plasmid promoter.

[0057] Fig. 17 is the construction of recombinant plasmid HindIII: pRSVneo in the opposite transcription orientation relative to the plasmid promoter.

[0058] Fig. 18 is the construction of recombinant plasmid BamHI: pRSVneo in the same transcription orientation relative to the plasmid promoter.

[0059] Fig. 19 is the construction of recombinant plasmid BamHI: pRSVneo in the opposite orientation relative to the plasmid promoter.

[0060] Fig. 20 is a schematic illustration of the RSV vector family.

[0061] Fig. 21 is a schematic illustration of recombinant plasmids pRSVgpt including the polynucleotide constructs of the present invention in both orientations relative to the plasmid promoter.

[0062] Fig. 22 illustrates multiple polynucleotide constructs for the retrovirus HIV.

[0063] Fig. 23 illustrates multiple polynucleotide constructs for the retrovirus FeLV.

[0064] Fig. 24 illustrates multiple polynucleotide constructs for the retrovirus HTLV. I.

[0065] Figs. 25 and 26 are a graph and chart illustrating data obtained from Feline Leukemia ELISA tests with Feline leukemia virus in a 1:5 dilution.

[0066] Figs. 27 and 28 are a graph and chart illustrating data obtained from Feline Leukemia ELISA tests with Feline leukemia virus in a 1:10 dilution.

[0067] Figs. 29 and 30 are a graph and chart illustrating data obtained from Feline Leukemia ELISA tests with Feline leukemia virus in a 1:20 dilution.

[0068] Figs. 31 and 32 are a graph and chart illustrating data obtained from Feline Leukemia ELISA tests with Feline leukemia virus in a 1:50 dilution.

[0069] Figs. 33 and 34 are Southern blot-hybridization results using ribo probes Neo and Bam respectively.

[0070] Many of the procedures useful for obtaining the cells of the present invention, whether or not described herein in detail, are well known to those skilled in the art of Recombinant DNA technology. A detailed description of many of such procedures can be found in Maniatis et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1982).

[0071] The present invention specifically involves the inhibition of retroviral replication by RNA complementary or homologous to essential replication sites in the retrovirus genome and proteins. In the following examples, the methods of the present invention are applied to HIV, HTLV-1, and FeLV viruses for purposes of illustration of the invention taught herein and are not limited thereto. The nucleic acid sequences of the Long Terminal Repeat region of HIV, HTLV-1 and FeLV are shown in Figs. 2, 3 and 4, respectively. Table 1 lists several sequences

which are exemplary of the polynucleotides employed in practicing the present invention, see Fig. 5. Use of these sequences is not limited to the HIV virus but can apply in accordance with the methods described herein to all retroviruses, although some changes in specific bases within the polynucleotide may be required. The genetic code is degenerate and redundant, that is, numerous substitutions, deletions, inversions or insertions of nucleotides will code for the same end product, i.e., protein. Consequently, it will be apparent that any changes or modifications to a given polynucleotide that produce a new polynucleotide that either retains the ability to code for the same end product i.e., protein, or retains sufficient homology to hybridize to targeted nucleic acid sequences within the retroviral genome so as to inhibit retroviral replication are functional equivalents of specific sequences disclosed herein.

[0072] The gene construct is a double-stranded DNA oligonucleotide sequence operatively linked to a Pol III promoter and terminator. The gene construct codes for a transcribed anti-sense RNA molecule complementary

Example 1

[0072] The gene construct is a double-stranded DNA oligonucleotide sequence operatively linked to a Pol III promoter and terminator. The gene construct codes for a transcribed anti-sense RNA molecule complementary

to a nucleic acid segment within the retrovirus genome essential to retrovirus replication including the LTR the AUG start codon regions, the U5 region, and the U3 region.

[0073] Unmodified polynucleotides were synthesized on an automated DNA synthesizer (Biosearch, San Rafael, California) using standard phosphoramidite chemistry. Gait, M.J. ed., Oligonucleotide Synthesis, IRL Oxford; Biosearch, Inc., Instruction Manual Model 8600, San Rafael, Ca., (1984). Deblocking of the support bound 5'-DMT group is removed with dichloroacetic acid to generate free 5'-OH for coupling. The 5'-OH is treated with a mixture of amidite and activator. This coupling results in a formation of a new phosphorus-oxygen bond which increases the length of the polynucleotide by one base unit. Because the dichloroacetic acid used in the deblocking step can break the phosphorous (III)-oxygen bond, it is necessary to oxidize the phosphorous (III) to a stable Phosphorus (V) oxidation state.

[0074] Iodine is used in this oxidation procedure. The unreacted 5'-OH groups are capped to prevent further reaction. Ammonia hydroxide cleaves the synthesized polynucleotide from the support. The cyanoethyl groups on the phosphorus, the benzoyl and isobutyl groups on the primary amino groups of the bases are removed from the DNA fragment by treating the collected ammonium hydroxide solution at 50°C for 5 hours. The polynucleotides were purified using 5% polyacrylamide gel electrophoresis. Maxam et al, P.N.A.S., 74: 560-564 (1977). The novel gene constructs have a perfered range from about 25 to 200 bases in length. The constructs were assembled from polynucleotides 30 to 45 bases in length. The short strand polynucleotides were constructed with sticky ends and complementary to each other so the complementary fragments would hybridize to form duplexes. The duplexes were then ligated together at the sticky ends using a ligation mixture of T4 DNA ligase, 10x ligase buffer, 10 mM ATP, and distilled water in a water bath at 50°C to form a double-stranded DNA fragment.

[0075] Polynucleotides complementary to the 3'R region (herein after Anti-R); the primer binding region (herein after Anti-PBS); the splice sites (herein after Anti-S); the cap site (herein after Anti-cap) and the first AUG start codon region (herein after Anti-AUG) in the Long Terminal Repeat region of the retroviruses HIV and FeLV were synthesized. Polynucleotides complementary to the above sites will also be synthesized for HTLV-I. Note that the Anti-PBS constructs are identical for HTLV-I and FeLV since their genomic sequences are homologous. The sequences of these polynucleotides are shown in Figs. 6-14. To derive the specific Anti-sense strand it was necessary to start with the RNA sequence of the desired retrovirus. Then, the homologous DNA strand is designed from the RNA strand by replacing the Uracil nucleotides with Thymine. To obtain the Anti-sense strand, the DNA sequence is inverted in order and the complementary sequence is designed. This yields

the exact nucleotide sequence to be synthesized.

[0076] The polynucleotide constructs are of sufficient length to insure stability and prevent degradation by host cell enzymes. But, the length associated with the constructs herein reduces the problem of the formation of tertiary structures associated with long strand polynucleotides which fold or "hairpin" thus preventing binding of the fragments to the retrovirus nucleic acid fragment.

[0077] The gene constructs were further constructed with flanking restriction sites at the 5' and 3' ends. Each polynucleotide was synthesized with a HindIII and a BamHI site at either end to allow insertion in both orientations. The nucleotide sequence for the HindIII site is "AAGCTT"; the nucleotide sequence for the BamHI site is "GGATCC".

[0078] A vector was used to introduce the gene constructs into a cell. "Vector" specifically refers to a flanking nucleic acid sequence which will allow the synthetic polynucleotide to be introduced into a cell and then either inserted into a chromosome or replicated autonomously. Certain vectors, e.g., plasmids, may also be used as a means to amplify the constructs of the present invention. The plasmids pRSVneo, pSV2gpt, pSV2neo, pUC19, pRSVcat and pRSVgpt were used as vectors for the preferred constructs of the present invention. Plasmids are circular pieces of DNA. They generally have a bacterial origin of replication and a selectable marker gene appropriate for amplification within a bacterial host such as E. coli. Plasmid pRSVneo has a marker gene which confers resistance to ampicillin. Plasmid pRSVgpt has a marker gene which confers resistance to xanthine. Other vectors, including without limitation other plasmids, viruses and retroviruses can alternatively be used in practicing the present invention. The plasmids used herein were selected because they are well characterized, have good dominant selective markers when incorporated into a cell, and the restriction endonucleases HindIII and BamHI only cut once making it relatively easy to clone the novel gene constructs of the present invention into the plasmids.

[0079] The selected plasmids pRSVneo, pSV2gpt, pSV2neo, pUC19, and pRSVgpt were each linearized by digestion with conventional restriction endonucleases BamHI and HindIII respectively, purchased from Bethesda Research Labs. The restriction enzymes were added to plasmids at conventional concentrations and temperature and with buffers as recommended by Bethesda Research Lab. The digestion mixture consisted of Plasmid DNA, TE buffer (pH=8), 2X restriction buffer, and the restriction enzyme. The mixture was incubated in a 37° water bath for 3 hours. The digest was then applied to a 1% preparative agarose gel and electrophoresed for 1 hour at 100 volts for separating the cut plasmid fragments from the uncut supercoiled plasmids according to the standard procedure described in Maniatis et al, Molecular Cloning, (1982). The linearized plasmids were then ligated together at the restriction endo-

nuclease sites HindIII and BamHI respectively using a ligation mixture of T4 DNA ligase, 10x ligase buffer, 10 mM ATP, and distilled water in a water bath at 50°C to form a re-circularized double-stranded plasmid comprising the synthesized gene construct. Refer to Figs. 16-19 illustrating the construction of the pRSVneo plasmid with the gene construct inserted at either the HindIII restriction site (herein after HindIII construct) or the BamHI restriction site (herein after the BamHI construct). The recombinant pRSVneo plasmids are designated either pGB-neo-H (1) or (2); or pGB-neo-B (1) or (2). This signifies that the plasmid "p" was made by Greatbatch GenAid "GB", is the "neo" plasmid in the RSV vector family, inserted in either the HindIII or BamHI site and is either in the same orientation as the plasmid promoter which is designated "(1)" or in the opposite direction designated "(2)". Refer to Fig. 20 illustrating the RSV vector family. The figures show two illustrations for each restriction site because the gene constructs were cloned into the plasmids in both transcription orientations relative to the plasmid RSV promoter. The BamHI construct for FeLV was inserted at nucleotides 3393 through 3678 in the pRSVneo plasmid. The HindIII construct for FeLV was inserted at nucleotide 5736 through 5921 in the pRSVneo plasmid. The BamHI construct for HIV was inserted into the plasmid pRSVgpt at nucleotides 3393 through 3733. The HindIII construct for HIV was inserted into the plasmid pRSVgpt at nucleotides 5736 through 6076. The difference between plasmid constructs pRSVgpt and pRSVneo is pRSVgpt has the gpt gene instead of the neo gene as shown in Fig. 21. The pRSVgpt recombinant plasmids are designated in the same manner as the pRSVneo plasmids except neo is replaced with gpt.

[0080] *E. coli* cells were transformed with the modified plasmids comprising the gene constructs for the purpose of amplifying the plasmids. Cells of *E. coli* HB101, to be transformed with the plasmids pRSVneo and pRSVgpt was grown in L broth at 37°C with shaking to an OD₅₅₀ = 0.5 (5 x 10⁷ cells/ml). 3ml's of cells were chilled on ice for 10 minutes and harvested by centrifugation in a Sorvall rotor for 5 minutes at 4,000 rpm at 4°C. Cells were resuspended in 1/2 original volume in solution of 50 mM CaCl₂ and 10 mM Tris. Cl (pH 8.0), incubated on ice for 15 minutes, centrifuged at 4,000 rpm for 5 minutes at 4°C. The cells were resuspended in 1/15 of original volume in solution of 50mM CaCl₂ and 10 mM Tris. Cl (pH 8.0). .2 ml aliquots were dispensed into chilled tubes, and stored at 4°C for 12-24 hours. 0.4 mg of plasmid DNA in ligation buffer were added to the cells. The cells were then incubated on ice for 30 minutes, and transferred to a waterbath at 42°C for 2 minutes. 1.0 ml of L broth were added to each tube and incubated at 37°C for 30 minutes.

[0081] The transformed cells were selected for by spreading the cells on rich plates containing 2xYT medium, 0.15% Bacto-agar (Difco) and 20 mg/ml ampicillin for pRSVneo and xanthine for pRSVgpt. The trans-

formed *E. coli* HB101 cells carrying the respective plasmids were amplified by growing the cells with shaking at 37°C in 1 liter LB medium to an OD₆₀₀f.6. 2.5 ml of chloramphenicol (34 mg/ml in ethanol) were added, and

- 5 vigorous shaking applied for 12 to 16 hours. To isolate the amplified plasmid DNA, one or more liters of cells of *E. coli* HB101 were harvested by centrifugation at 4,000 rpm for 10mm in a Sorvall rotor and then resuspended in 10 ml of a solution containing 50 mM glucose, 25 mM Tris-CL (pH 8), 10 mM EDTA, 5 mg/ml lysozyme. The cells were left at room temperature for 5 minutes. 20 ml of Solution II containing 12 N NaOH, 1% SDS were added to the cells and then incubated on ice for 10 minutes. 15 ml of a cold solution of 5M potassium acetate (pH 15 4.8) were added to the cell solution, mixed by inversion and incubated on ice for 10 minutes. The cell lysis were centrifuged for 20 minutes at 4°C, at 20,000 rpm on a Beckman centrifuge. The plasmid DNA in the supernatant was precipitated with 0.6 volumes of isopropanol at 20 room temperature for 15 minutes. The plasmid DNA was recovered by centrifugation in a Sorvall rotor at 12,000 rpm for 30 minutes at room temperatures. The pellet was washed with 70% ethanol at room temperature, dried, and resuspended in total volume 8 ml of TE (pH 25 8.0). To purify the isolated plasmid DNA, the total volume of isolated plasmid DNA was measured. For every ml of plasmid DNA exactly 1 gram of solid cesium chloride was added, and mixed gently until the salt dissolved. To it was added 0.8 ml of ethidium bromide (10 mg/ml in H₂O) solution for every 10 ml of cesium chloride solution, mixed to yield a final density of 1.55 g/ml, and final concentration of ethidium bromide of approximately 600 mg/ml. The cesium chloride solution was transferred to tubes suitable for centrifugation in a Beckman Type-50 or Type-65 rotor. The remainder of the tube was filled with paraffin oil and then centrifuged at 45,000 rpm for 36 hours at 20°C. Two bands of DNA were visible, an upper band consisting of linear bacterial DNA and circular plasmid DNA, a lower band consisting of closed circular plasmid DNA. The lower band of plasmid DNA was collected by side puncture with a #21 hypodermic needle. The ethidium bromide was removed by adding equal volumes 1-butanol saturated with water, then mixed and centrifuged at 1,500 rpm for 3 minutes at room temperature. The aqueous lower phase was transferred to a clean tube and the above step repeated until the pink color disappeared. The aqueous phase was dialyzed with TE (pH 8.0). The preceding procedures are applicable to any gene construct cloned into any suitable plasmid in accordance with the methods taught herein.

- [0082]** An effective method of delivering the vector DNA into the target cell is required if high efficiency transformation is to be achieved. Transformation of potential host cells was carried out by CaPo₄ precipitation by standard procedures in the state of the art. Other suitable transformation methods are applicable herein and can be substituted. See Ausubel et al, Current Protocols

in Molecular Biology, (1987), which is incorporated herein by reference. For example, electroporation can be expected to increase plasmid transformation efficiencies. Transformation of HUT-78 cells i.e., a suitable host cell system for the HIV virus, was accomplished using a pRSVneo plasmid (without gene constructs) using electroporation at 1000V (2000v/cm) and a 14 mf capacitor, which gave a pulse length of 1.9 ms. This is an energy of 1/2 CE² or 7 joules. The HUT-78 cells were challenged with neomycin which demonstrated that the plasmid had indeed been transformed and expressed. In addition, an alternative method for delivering plasmid DNA into target cells which is believed to be particularly well suited to the treating of AIDS patients involves a) encapsulating plasmid DNA into liposomes, using established methods, b) binding commercially available antibodies to the surface of the liposomes which specifically bind to T4 cells, and c) injecting these liposomes into the blood stream of the AIDS patient. The antibody-targeted liposomes will bind to T4 cells, and will continually be absorbed by the cell, leading to transformation. Subsequently, transformed T4 cells will have a selective advantage over non-transformed cells, due to their immunity to the HIV virus. Such resistant cells will multiply as susceptible cells are killed off by the virus. This selection process can be enhanced by use of selective agents (i.e., antibiotics) favoring the transformed cells. Also, irradiation of the patient prior to injection of targeted liposomes could further reduce the number of infected T4 cells. This technique is well known to those skilled in the art of treatment of infant leukemias.

[0083] In accordance with the CaPO₄ precipitation procedure, a DNA slurry was prepared with 1 ml HBS buffer, 10 ml DNA sample (1 ug/ml), 18 to 25 μ l 2.5 M CaCl₂, added one drop at a time and vortexed. The remaining CaCl₂ was added and let stand at room temperature for 5 minutes. A media containing DME and mink lung cells was aspirated from dishes, and washed with HBS buffer. Then 500 μ l DNA slurry sample and 4ml of media containing the mink lung cells was added to each dish and incubated for 4 hours at 37°. The media was removed and 2 ml of 15% glycerol was added. The media was incubated for 3 minutes at 37° and then the glycerol was removed by aspirating. 4 ml of fresh media was added and the media was incubated at 37° for 48 hours. The transformed cells were selected by spreading the cells on a selective media depending on the particular plasmid i.e., ampicillin or xanthine, and incubated for two days at 37°C. G418 was the selective media for the ampicillin resistant plasmid pRSVneo. HAT media was the selective media for the xanthine resistant plasmid pRSVgpt. The transformed cells were the colonies that grew on the selective media after several weeks. Northern blot-hybridization analysis and Southern blot-hybridization analysis were used to verify the presence of high levels of RNA or DNA in the transformed cells. The probes for the Southerns and Northerns were constructed using ribo probes and in vitro transcription with

T7 RNA polymerase. Figs. 33 and 34 show representative Southern blot-hybridization analysis to verify the presence of the plasmid comprising the gene construct within the transformed mink lung cells. Fig. 33 shows a

- 5 blot analysis probing with ribo probe neo. This test result indicates that the plasmid was taken up by the mink lung cells during transformation and expressed by the cells. Plasmids pRSVneo:FeLV BamHI, nos. 8, 3-1; 8, 3-2 and 13-1 in rows 11, 12 and 13 respectively show bands measuring about 2485 kb indicating the presence of the plasmid. Fig. 34 shows a blot analysis probing with ribo probe BamHI. This test result indicates that the gene construct was present in the plasmid taken up by the mink lung cells during transformation. Plasmids
- 10 pRSVneo:FeLV BamHI, nos. 13-1; 8, 3-2 and in rows 11 and 12 respectively show bands measuring about 6021 kb indicating the presence of the gene construct in the expressed plasmid.
- 15

- [0084]** It should be noted that further modifications of 20 the novel gene constructs were carried out during the preparation of the constructs in accordance with the usual procedures. It is apparent that the gene constructs have an efficient promoter to express the gene in the relevant host cell. If the gene constructs were simply inserted into the plasmids employed herein, the gene would be expressed by the plasmid Polymerase II (herein after Pol II) promoter. Since the Pol II promoter is next to the HindIII site and distal from the BamHI site, the effect of this promoter on the gene construct is not 25 known.

- [0085]** Further the size of the gene fragment transcribed could not be predicted or regulated since the 30 promoter would not be responsive to a termination signal on the gene construct. Also, promoters vary greatly 35 in their promoter strength, and many promoters are regulated such that they are only "on" when induced by some stimulus.

- [0086]** The gene constructs incorporate an RNA 40 Polymerase III (herein after Pol III) promoter. The gene constructs incorporate a "box A" and a "box B" which comprise the Pol III promoter sequence. By constructing the gene constructs with an independent promoter, the genes can be transcribed independently of the plasmid promoter; therefore, the unregulated promoter should 45 express constitutively in all animal cells or tissues. By using a Pol III promoter the gene size is greatly reduced. This simplifies gene synthesis and allows the regulation of the amount of RNA produced simply by regulating the number of copies of the gene inserted into the vector.
- 50 Further, Pol III promoters tend to be more or less universal in their expression and should function equally well in a wide range of host cell systems. Also, such promoters do not appear to have "enhancer" activity which are potentially carcinogenic.

- [0087]** The actual promoter sequence could have different embodiments, and is not limited herein by the previous description. For example, the promoter region including the upstream region, the transcription initiating

region, "Box A" and "Box B" can be taken directly from any highly active, natural tRNA. A tRNA promoter sequence which has been shown to be particularly strong is the Glu tRNA gene in mouse. The Glu tRNA gene has the advantage that it is straight forward to use as an active promoter and the short tRNA sequence which will be transcribed should not have any effect on the activity of the gene construct.

[0088] Another promoter sequence could involve upstream sequences from the promoter coming from a natural tRNA gene such as Glu tRNA, while transcriptional initiation sequences "Box A", "Box B", and all intervening sequences could be supplied by the anti-sense RNA sequence itself with only relatively few base-pair substitutions. This has the advantage of economy and size which will facilitate synthesis and will allow maximum number of genes per vector. In addition, this promoter begins transcription precisely at the 5' end of the gene construct and ends transcription within several base pairs of the end of the gene construct sequence. This will be desirable where there is a need to minimize "extraneous" RNA sequences at the 5' and at the 3' ends of the resulting RNA molecule.

[0089] Such Pol III promoter constructs of the present invention should have a poly "T" site downstream from each Pol III promoter, i.e., which is the universal Pol III transcription termination signal. This site was included in the gene constructs to provide a transcription termination mechanism.

[0090] No molecular mechanism occurs with 100% efficiency. Furthermore, even those molecular mechanisms which normally have a very high efficiency can have poor efficiency under new circumstances. Therefore, optimal resistance will be achieved where more than one molecular mechanism is involved. Once a retrovirus has successfully inserted a single copy of its genome into a host's chromosome, there is no way to destroy it, except to destroy the cell. Therefore, it is especially desirable that the probability of this event can be reduced to an extremely low level. In certain alternative embodiments multiple polynucleotides were inserted into the same plasmid for transformation into a host cell system. The gene constructs were constructed with multiple Pol III promoters e.g., "box A" and "box B", and polynucleotides inserted between the promoters.

[0091] Figs. 22-24 show the gene sequences of the polynucleotide/promoter combinations employed in the present invention using this multiple attack approach for the retroviruses HIV, HTLV-1, and FeLV, respectively. Fig. 15 illustrates the multiple gene constructs employed in the present invention utilizing the specific sequences shown in Figs. 22-24, multiple promoters i.e., Box A and Box B, the Pol III terminator sequence, and either a BamHI or HindIII end for insertion into the respective restriction site. The gene constructs have additional restriction endonuclease sites for insertion of further anti-sense producing gene fragments. Thus the gene constructs will transcribe multiple short anti-sense frag-

ments complementary to multiple essential hybridization sites within a retrovirus genome. This will affect the retrovirus at multiple hybridization sites which is believed to have a synergistic effect in inhibiting retrovirus growth.

[0092] To illustrate the inhibition effect of the gene constructs in accordance with the present invention, transformed mink lung cells were challenged with Feline Leukemia Virus. The transformed cells were harvested under appropriate nutrient conditions. DME with 0.5 ug/ml of polybrine was mixed with the harvested cells to obtain the desired dilution of cells. The dilutions employed ranged from 1:5 to 1:50. The culture was incubated for 2 hours at 37°C and the media was replaced with an amount of fresh media equivalent to the desired dilution. The FeLV was added at the appropriate dilution with polybrine and incubated overnight. The media was aspirated and the cells washed with 5 ml of fresh media. Then, 10 ml of DME only was added to each dish. The media was incubated with aliquots removed daily for testing the amount of virus present in the media. The ELISA test was used according to standard procedures in the art to test the amount of FeLV present in the media. Synbiotics Corp., Feline Leukemia Virus Test Kit, San Diego. A spectrophotometer reading was taken to quantify the results. The resulting data is summarized in Figs. 25-32. Note, refer to Figs. 16-19 for the construction of the Recombinant plasmids. The 1:5 dilution of FeLV in Figs. 25 and 26 show that the mink lung cells, including the plasmid comprising the polynucleotide constructs, when challenged with the FeLV virus show an accumulation of virus ranging from 0.13 to 0.31 after seven days. This is significantly lower than the control of mink lung cells without the plasmid comprising the polynucleotide constructs showing an accumulated virus level of 0.56 after four days. These results show a significant decrease in accumulated FeLV virus levels when the mink lung cells are transformed with the gene constructs in accordance with the procedures and methods of the present invention. The tables indicate gene constructs "GB" with various labels such as pGB-neo-B-#8-3-1. This signifies that the gene construct was made by Greatbatch GenAid and inserted into the pRSVneo plasmid at the BamHI restriction site. Plasmid #8 was selected for transformation and transformed cells 3-1 were chosen to be challenged with FeLV.

[0093] The 1:10 dilution of FeLV in Figs. 27 and 28 show that the mink lung cells, including the plasmid comprising the polynucleotide constructs, when challenged with the FeLV virus show an accumulation of virus ranging from 0.01 to 0.28 after six days. This is significantly lower than the control of mink lung cells without the plasmid comprising the polynucleotide constructs showing an accumulated virus level of 0.46 to 0.51 after six days. These results show a significant decrease in accumulated FeLV virus levels when the mink lung cells are transformed with the gene constructs in accordance with the procedures and methods of the present invention.

tion.

[0094] The 1:20 dilution of FeLV in Figs. 29 and 30 show that the mink lung cells, including the plasmid comprising the polynucleotide constructs, when challenged with the FeLV virus show an accumulation of virus ranging from 0.08 to 0.14 after seven days. This is significantly lower than the control of mink lung cells without the plasmid comprising the polynucleotide constructs showing an accumulated virus level of 0.36 after six days. These results show a significant decrease in accumulated FeLV virus levels when the mink lung cells are transformed with the gene constructs in accordance with the procedures and methods of the present invention.

[0095] The 1:50 dilution of FeLV in Figs. 31 and 32 show that the mink lung cells, including the plasmid comprising the polynucleotide constructs when challenged with the FeLV virus show an accumulation of virus ranging from 0.045 to 0.06 after seven days. This is significantly lower than the control of mink lung cells without the plasmid comprising the polynucleotide constructs showing an accumulated virus level of 0.14 after six days. These results show a significant decrease in accumulated FeLV virus levels when the mink lung cells are transformed with the gene constructs in accordance with the procedures and methods of the present invention.

Example 2

Human Bone Marrow Auto-Transplant

[0096] This embodiment is in accordance with the procedures and methods described in Example 1. Auto-transplant of bone marrow (i.e., re-introducing a patient's own bone marrow cells) is now a relatively minor and routine procedure. Bone marrow cells are extracted by syringe, and in the present case, transformed and cultured. The patient is then irradiated, or otherwise treated, to destroy existing bone marrow cells remaining in the patient. Then the transformed cultured bone marrow cells are injected back into the patient's circulatory system. Such cells eventually migrate back into the bone marrow, re-establishing that tissue.

[0097] In order to make all of the lymphocytes of a patient immune to the AIDS-related virus, bone marrow cells would be transformed with the gene constructs of the present invention by any of the methods already discussed, and transformed cells would be selected. Multiple gene constructs can be employed. Preferably, the polynucleotide sequence(s) employed in the constructs will direct transcription of RNA complementary or homologous to the R region, the primer binding site (PBS) with a false leader and a false primer coding sequence, the first splice region and/or the AUG site. Non-transformed bone marrow within the patient would be destroyed and the transformed bone marrow cells would be used to re-establish the bone marrow tissue. As a

result, all lymphocytes, including T4 cells, deriving from the transformed bone marrow would be immune to the virus. Consequently, the virus would be eradicated from the patient's system. It should be noted that irradiation of the patient may not be necessary since the AIDS pathology results in killing of infected cells by the HIV virus itself thus reducing the infected T4 cell population.

[0098] Auto-transplant can be performed in both human and other mammalian, e.g., feline systems. In cats, the germline of the animal might be transformed to produce virus resistant strains (breeds) of cats.

Example 3

Injection of Transformed Liposomes

[0099] Alternatively, transformation can be accomplished *in situ* by encapsulating constructs of the present invention into liposomes using established methods, binding commercially available antibodies to the surface of the liposomes which specifically bind to T4 cells, and injecting these liposomes into the blood stream of the patient. The antibody-targeted liposomes will bind to T4 cells, and will continually be absorbed by the cell, leading to transformation. Subsequently, transformed T4 cells will have a selective advantage over non-transformed cells, due to their immunity to virus. Such resistant cells will multiply as susceptible cells are killed off by the virus. This selection process can be enhanced by use of selective agents (i.e., antibiotics) favoring the transformed cells. This method would be especially appropriate for AIDS patients who could not tolerate a bone marrow transplant (see above).

[0100] This embodiment is in accordance with the procedures and methods described in Example 1, and can utilize multiple genes in multiple copies, with Pol III promoters. Preferably, the polynucleotide sequence(s) employed in the constructs will direct transcription of RNA complementary or homologous to the R region, the primer binding site, the first splice site region and/or AUG start codon region.

Example 4

Blocking of Reverse Transcription using an Anti-sense RNA Molecule Complementary to the "R" Region.

[0101] This embodiment is in accordance with the procedures and methods described in Example 1. As already described, the R region, found at both ends of the retroviral RNA genome, plays a crucial role in the "first jump" of reverse translation. Reverse transcription becomes stalled at the 5' end of the virus and must be carried to a new template at the 3' end of the virus. This is possible because the enzyme is attached to the cDNA strand which has been transcribed from the 5' R region. This cDNA is naturally complementary to R and can hybridize to the 3' R region. This results in a bridge which

circularizes the virus and allows reverse transcription to continue.

[0102] This "first jump" can be blocked by an independent RNA construct which is complementary to the R region. This construct is referred to as "Anti-R". This molecule can hybridize to the 3' R region and will block the 5'-3' bridge from forming between the two ends of the virus. Such hybridization tends to be stable, such that competition for the R hybridization site is on a first-come basis. Since reverse transcription is a particularly slow process, the cDNA molecule transcribed from the 5' end of the virus will not be available until some time after initial cell infection. Therefore, if abundant independent RNA constructs are already present in the cell, and are complementary to R, there will be a very high probability that the R site will be blocked (bound) by them, before the "first jump" is even possible. Consequently, this will preclude successful infection of the cell by that viral strand. Refer to Figs. 6-8 for the sequence of the Anti-R gene constructs of the HTLV-1, FeLV, and HIV viruses respectively.

[0103] The R region of retroviruses is the most highly conservative (unchanging) region. However, point mutations do occur in this region. Different R region sequences of different HIV strains show several minor nucleotide differences in this region. Newly arising mutant strains will also have small differences in this region. This is not an important consideration, since nucleic acid hybridization does not require perfect base-pairing. Likewise, the interfering molecule may have additional sequences 5' and 3', or may be less than full length at the R region. The relevant point is that any such gene constructs as herein described stably hybridize to the R region of the virus, even though homology is not complete.

Example 5

Blocking the Primer Binding Site (PBS) and Adjacent Sequences Using Anti-Sense RNA. (Example for better understanding the invention)

[0104] This embodiment is in accordance with the procedures and methods described in Example 1. This gene construct will bind to the primer binding site region (herein after "Anti-PBS"). As a result, it will compete with the tRNA(Lys)/reverse transcriptase samples for this site, thereby blocking initiation of reverse transcription. In addition, it will bind to the double-strand DNA which is involved in the "second jump" of reverse translation. This will block completion of reverse translation in a similar way as the first two anti-viral molecules. Because of complementation to the 3' end of the U5 region, this construct may also affect circularization and insertion into the chromosome of the double-stranded viral DNA. Refer to Figs. 9-11 for the sequences of the Anti-PBS gene constructs of the FeLV, HTLV-1 and HIV viruses respectively.

Example 6

Anti-Sense RNA Complement to the Poly-Purine Sequence and Adjacent Sequences. (Example for better understanding the invention)

[0105] This embodiment is in accordance with the procedures and methods described in Example 1. This gene construct includes the 5' end of the U3 region. It is complementary to the single-strand DNA, in the region where second strand DNA synthesis begins. If in a RNA form, and if associated with improper flanking sequences, this molecule will bind in the initiation region of second strand DNA synthesis and will block proper synthesis of the double-strand DNA.

Example 7

Blocking of the First RNA Splice Site and the First AUG Start Codon Site.

[0106] This embodiment is in accordance with the procedures and methods described in Example 1. Many RNA molecules must have intervening sequences removed or "spliced out" before they can be properly translated into protein. The HIV virus has at least four "splice" sites. Splicing at such sites is required for translation of several proteins downstream of GAG. Such splicing involves precise recognition of RNA single-stranded sequences by proper enzymes. Complementary RNA gene constructs, hybridizing in such regions will prevent proper splicing and thereby prevent proper protein translation (herein after "Anti-SD"). Near the same region is the first AUG site, where translation of GAG protein begins. This site is also subject to hybridization interference, since protein translation can not be initiated in a region of double-stranded RNA (herein after "Anti-AUG"). Therefore, complementary RNA molecules spanning these two regions will block translation of GAG protein as well as the other proteins further downstream. Refer to Figs. 12-14 for the Anti-AUG and Anti-S sequences of FeLV, HTLV-1, and HIV viruses.

[0107] As will be seen the RNA molecules already described can be predicted to have additional anti-HIV activity by interfering with additional viral mechanisms. The multi-functional nature of these molecules is important in establishing multiple lines of defense. These are described below:

Example 8

Blocking Circularization and Chromosomal Insertion.

[0108] This embodiment is in accordance with the procedures and methods described in Example 1. The precise mechanisms involved in circularization and chromosomal insertion are unknown, although the 5' and the 3' ends of the virus are obviously involved. The

short inverted repeats at these ends presumably allow end-to-end hybridization. It is noteworthy that certain complementary gene constructs described herein include the inverted repeat at the 5' end of the DNA virus, and the inverted repeat at the 3' end of the DNA virus. Therefore, gene constructs already described provide potential interfering mechanisms for the viral insertion processes.

Example 9

Blocking DNA Transcription/Translation.

[0109] This embodiment is in accordance with the procedures and methods described in Example 1. Some retroviruses, like HIV, have a specific open reading frame which codes for a transcriptional activator TAT protein. In the absence of this protein, the pro-virus is not transcribed and/or translated (has been controversial), and all viral activities cease. Translation of this protein will be blocked by previously described gene constructs. Specifically, the Anti-SD and the Anti-AUG gene constructs can be targeted to block synthesis. Consequently gene constructs already described can be used to block transcription and/or translation by blocking synthesis of the transcriptional activator protein.

Example 10

Blocking of the RNA Packaging Site.

[0110] This embodiment is in accordance with the procedures and methods described in Example 1. A region of the viral RNA that is essential for packaging of the RNA into infectious particles has been shown in other retroviruses to be between the first splice site and the GAG coding region. It appears that this region binds to one of the GAG proteins. This region is included in the region complemented by the Anti-SD and the Anti-AUG gene constructs. Therefore, the previously described constructs are expected to block RNA packaging, as well as blocking RNA splicing and translation.

Example 11

Blocking the poly-A Attachment Site

[0111] This embodiment is in accordance with the procedures and methods described in Example 1. Retroviral RNA is normally processed, like other mRNA's, by enzymatic splicing of a poly-A tail on the 3' end of the molecule. This is considered important for transport of the RNA out of the nucleus, and for stability in the cytoplasm. The previously described "anti-R" gene construct should bind to the poly-A attachment site and interfere with such RNA processing in this region.

Example 12

Blocking of Dimer Formation and Genomic Folding.

- 5 **[0112]** This embodiment is in accordance with the procedures and methods described in Example 1. Infectious retroviral particles contain two identical RNA genomic molecules which have regions of mutual hybridization i.e., dimer formation. They have as well, regions of internal hybridization and folding within each molecule. The exact role of these 3-dimensional configurations is unclear, but they appear to be universal and therefore important. The area of dimerization between the two molecules is in the U5 wet region. Therefore the
 10 previously described gene constructs should block dimer formation and should interfere with internal hybridization and folding, within the individual molecules.
[0113] While the above-mentioned anti-viral molecules are simple complements of different regions of the retroviruses, more complex gene constructs can be employed to enhance anti-viral activity. These are described below:

Example 13

Compound Genes

- [0114]** This embodiment is in accordance with the procedures and methods described in Example 1. Compound gene constructs can be synthesized which will code for mRNA consisting of tandem repeats at the same anti-viral sequence, or chimeric mRNA's containing more than one anti-viral sequence. By this method, the same promoter can transcribe proportionately more
 30 anti-viral material. Chimeric mRNA may have the added anti-viral trait of cross-linking different parts of the virus, disrupting genomic structure and function.

Example 14

False Templates

- [0115]** This embodiment is in accordance with the procedures and methods described in Example 1. The reverse transcriptase enzyme of the virus can be used against itself, through the use of false templates. As already mentioned, certain RNA sequences serve as initiation sites for reverse transcription (i.e., the primer binding site, herein after PBS), or serve as re-initiation
 45 sites for reverse transcription (i.e., the R region, and the PBS region, at the first and second jump events, respectively). Reverse transcription normally begins from these sequences, and any sequence 5' from these sequences will be automatically reverse transcribed. By adding inappropriate sequences 5' to the R and PBS sites as previously described, false templates are created. These false templates have the original anti-viral
 50 activities of the R and PBS molecules. In addition, they
 55

have several new properties: a) In the case of initiating reverse transcription, the PBS false template will bind and "disarm" reverse transcriptase complexes; b) In terms of re-initiating reverse transcription after the first and second jumps, false templates will lead the reverse transcription process down a "false path", leaving the original template destroyed, and the new cDNA abortive; c) Because the false templates will be reverse-transcribed, cDNA complementary to the 5' end of the molecule will be created by the reverse transcriptase enzyme. The resulting DNA sequence may have still further anti-viral activity. For example, if the 5' RNA sequence employed in the false template was a complement of the poly-purine tract, the resulting cDNA would be the DNA equivalent of the poly-purine tract, which would stably bind to the initiation site for second strand DNA synthesis, blocking correct initiation of DNA synthesis from this point.

Example 15

False Primers.

[0116] This embodiment is in accordance with the procedures and methods described in Example 1. False primers can be created by placing a lysine tRNA sequence at the 5' end of any of several of the types of gene constructs already discussed. The result will be a modified lysine tRNA, which will have lost its original site for binding to the PBS. Instead, the modified lysine tRNA will bind at a different part of the viral genome, as dictated by the specific complementary "tail" selected. Consequently, the resulting false primer will complex with reverse transcriptase enzyme, and will initiate reverse transcription at an improper site. This will cause the viral template to be progressively degraded from that point, and will result in abortive and non-infectious cDNA with improper "ends" required for circularization and insertion.

[0117] While most anti-sense gene constructs involve Hybridization Interference between nucleic acids, there are a few resistance mechanisms involving protein gene products. These are described below for HIV:

Example 16

Creation of a Transcriptional or Translational Repressor Protein Specific for the HIV R Region.

[0118] This embodiment is in accordance with the procedures and methods described in Example 1. The TAT protein has been shown to be essential to HIV translation and replication, by binding to the R region. The TAT protein, if modified, could be changed into a repressor protein, which would bind the same site, but would block translation rather than induce it. This would be done by sub-dividing the protein into its functional domains, and deleting the activator domain or mutating it,

or by sub-cloning the domain responsible for recognizing and binding to the viral R region. For example, the arginine-rich region in the second half of the activator protein sequence is a likely region, important in the RNA binding process.

- 5 The second half of this protein sub-domain might adequately compete with the fully functional protein for the binding site, thereby acting as a repressor. In addition, bulky DNA interactive amino acid chains might be added to the DNA-binding sub-domain, to further interfere with initiation of translation or transcription in this region of RNA or DNA.

Example 17

Creation of a self-destruct gene.

- [0119] This embodiment is in accordance with the procedures and methods described in Example 1. The fact that the HIV virus is self-activating provides an excellent opportunity to engineer a "hyper-sensitive" form of resistance. By combining the natural HIV promoter and 'R' sequence with sequence coding for any type of lysis protein (which would come from a number of sources), a "self-destruct" mechanism is created for the cell.
- 20 Upon initial infection of the cell, the viral activator protein is produced, which will activate the HIV virus promoter, or initiate translation, leading to production of the lysis protein and destruction of the cell before the virus has an opportunity to reproduce. This mechanism might be suitable for use very early in infection, or after bone marrow substitution, or as a backup mechanism in conjunction with genes blocking the initial infection process.

Example 18

The ENV protein.

- [0120] This embodiment is in accordance with the procedures and methods described in Example 1. There is evidence that the gene of the ENV protein of retroviruses can condition resistance, if expressed constitutively in the cell. In this case, a host cell is transformed with a ENV gene construct. The resulting RNA is translated into ENV protein, which binds to, and saturates, the cell surface antigens of the cell. Because the binding sites are already saturated, viral particles can not bind to and infect the cell.

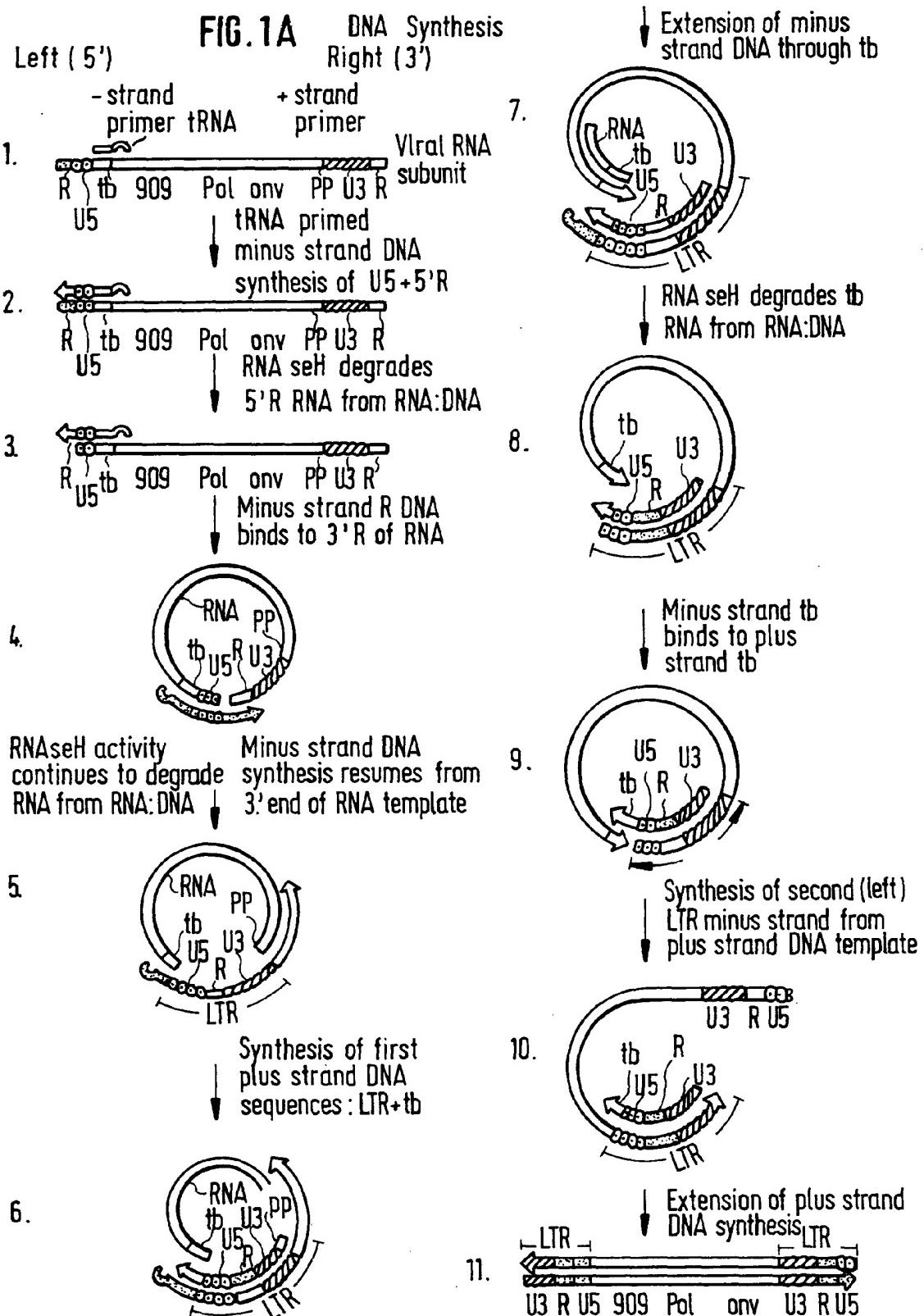
Claims

- 50 1. A cell modified by having resistance to infection by a retrovirus, said modification comprising that said cell contains a vector comprising a polynucleotide which is transcribed to RNA within said cell, said RNA being selected from the group consisting of RNA complementary to a nucleic acid sequence within at least one region within the genome of said

- retrovirus, wherein said region is selected from the group consisting of the LTR region, the U5 region, the U3 region, the 3'R region, and the AUG start codon region, thereby inhibiting, in the infection process, at least one process selected from the group consisting of retroviral replication, reverse transcription, and translation.
2. The cell according to claim 1, wherein said retrovirus is a virus selected from the group consisting of a human T-cell lymphotrophic virus, a human immunodeficiency virus, a lymphadenopathic virus, a leukemia virus, a sarcoma virus and a virus causing lymphotrophic disease.
3. A cell according to claim 1 or claim 2, wherein said retrovirus is a virus selected from the group consisting of HIV, feline leukemia virus, HTLV-I, HTLV-II, murine leukemia virus and avian leukemia virus.
4. The cell according to any of the claims 1 to 3, wherein said polynucleotide is a synthetic polynucleotide or wherein said polynucleotide is DNA.
5. The cell according to any of the claims 1 to 4, wherein said vector is selected from the group consisting of a viral vector, a retroviral vector and a plasmid.
6. The cell according to claim 5, preferably wherein said vector is a plasmid.
7. The cell according to claim 6, wherein said plasmid is selected from the group consisting of pUC19, pRSVcat, pSV2gpt, pSV2neo, pRSVneo and pRS-Vgpt.
8. The cell according to any of the claims 1 to 7, wherein said vector further comprises a first promoter which controls transcription of said RNA within said cell.
9. The cell according to any of claims 1 to 8, wherein said vector further comprises a first terminator which controls termination of transcription of said RNA within said cell.
10. The cell according to any of the claims 1 to 9, wherein said vector further comprises a marker for selection of transformed cells.
11. The cell according to any of the claims 1 to 10, wherein said polynucleotide is a polynucleotide directing transcription of a single RNA which is complementary to multiple hybridization sites within the retrovirus genome.
12. The cell according to any of the claims 1 to 11, wherein said polynucleotide is a polynucleotide fur-
- ther comprising a second promoter which controls transcription of said RNA within said cell.
13. The cell according to claim 12, wherein said promoter is RNA Polymerase III promoter.
14. The cell according to any of the claims 1 to 13, wherein said polynucleotide is a polynucleotide further comprising a second terminator which controls termination of transcription of said RNA within said host cell.
15. The cell according to claim 14, wherein said second terminator is RNA Polymerase III terminator sequence.
16. The progeny of the cells according to any of the claims 1 to 15 containing the vector defined in claim 1.
20. The progeny of the cells according to claim 16, wherein said polynucleotide, said vector, and said retroviral infection are defined as in any one or more of claims 2 to 15.
25. Cell and/or any progeny thereof according to any of claims 1 to 17, wherein said cell and/or said progeny contains the vector defined in claim 1, as a medicament.
30. Cell and/or any progeny thereof according to claim 18 as a medicament conferring resistance to retroviral infection upon cells.
35. Cell and/or any progeny thereof according to claim 18 as a medicament conferring upon cells resistance to infection by retroviruses selected from the group consisting of a human T-cell lymphotrophic virus, a human immunodeficiency virus, a lymphadenopathic virus, a leukemia virus, a sarcoma virus and a virus causing lymphotrophic disease.
40. Cell and/or progeny thereof according to claim 18 as a medicament conferring upon cells resistance to HIV infection.
45. Patentansprüche
50. 1. Zelle, dadurch modifiziert, daß sie Resistenz gegen Infektion mit einem Retrovirus aufweist, wobei die Modifikation umfaßt, daß die Zelle einen Vektor enthält, der ein Polynukleotid umfaßt, das innerhalb der Zelle in RNS transkribiert wird, wobei die RNS aus der Gruppe gewählt ist, die besteht aus RNS, die komplementär zu einer Nukleinsäuresequenz innerhalb wenigstens einer Region innerhalb des Genoms des Retrovirus ist, worin die Region ge-

- wählt ist aus der Gruppe, die besteht aus der LTR-Region, der U5-Region, der U3-Region, der 3'R-Region und der AUG-Startkodon-Region, wodurch im Rahmen des Infektionsprozesses wenigstens ein Prozeß inhibiert wird, der gewählt ist aus der Gruppe, die besteht aus Replikation des Retrovirus, reverse Transkription und Translation.
2. Zelle nach Anspruch 1, worin der Retrovirus ein Virus ist, der gewählt ist aus der Gruppe, die besteht aus Human-T-Zellen-Lymphotroph-Virus, Human-Immunodefizienz-Virus, Lymphadenopathie-Virus, Leukämie-Virus, Sarkom-Virus und Virus, das lymphotrophe Krankheiten verursacht.
3. Zelle nach Anspruch 1 oder Anspruch 2, worin das Retrovirus ein Virus ist, das gewählt ist aus der Gruppe, die besteht aus HIV-Virus, Katzen-Leukämie-Virus, HTLV-I, HTLV-II, Mause-Leukämie-Virus und Vogel-Leukämie-Virus.
4. Zelle nach einem der Ansprüche 1 bis 3, worin das Polynukleotid ein synthetisches Polynukleotid ist oder worin das Polynukleotid DNS ist.
5. Zelle nach einem der Ansprüche 1 bis 4, worin der Vektor gewählt ist aus der Gruppe, die besteht aus einem viralen Vektor, einem retroviralen Vektor und einem Plasmid.
6. Zelle nach Anspruch 5, worin vorzugsweise der Vektor ein Plasmid ist.
7. Zelle nach Anspruch 6, worin das Plasmid gewählt ist aus der Gruppe, die gewählt ist aus pUC19, pRSVcat, pSV2gpt, pSV2neo, pRSVneo und pRSVgpt.
8. Zelle nach einem der Ansprüche 1 bis 7, worin der Vektor weiter einen ersten Promotor umfaßt, der die Transkription der RNS innerhalb der Zelle steuert.
9. Zelle nach einem der Ansprüche 1 bis 8, worin der Vektor weiter einen ersten Terminator umfaßt, der die Beendigung der Transkription der RNS innerhalb der Zelle steuert.
10. Zelle nach einem der Ansprüche 1 bis 9, worin der Vektor weiter einen Marker zur Selektion transformierter Zellen umfaßt.
11. Zelle nach einem der Ansprüche 1 bis 10, worin das Polynukleotid ein Polynukleotid ist, das die Transkription einer einzelnen RNS steuert, die komplementär zu Mehrfach-Hybridisierungs-Stellen innerhalb des Genoms des Retrovirus ist.
12. Zelle nach einem der Ansprüche 1 bis 11, worin das Polynukleotid ein Polynukleotid ist, das weiter einen zweiten Promotor umfaßt, der die Transkription der RNS innerhalb der Zelle steuert.
- 5 13. Zelle nach Anspruch 12, worin der Promotor der RNS-Polymerase III-Promotor ist.
- 10 14. Zelle nach einem der Ansprüche 1 bis 13, worin das Polynukleotid ein Polynukleotid ist, das weiter einen zweiten Terminator umfaßt, der die Beendigung der Transkription der RNS innerhalb der Wirtszelle steuert.
- 15 15. Zelle nach Anspruch 14, worin der zweite Terminator eine RNS-Polymerase III-Terminator-Sequenz ist.
- 20 16. Nachkommenschaft der Zellen nach einem der Ansprüche 1 bis 15, enthaltend den Vektor, der in Anspruch 1 definiert ist.
- 25 17. Nachkommenschaft der Zelle nach Anspruch 16, worin das Polynukleotid, der Vektor und die Infektion mit einem Retrovirus wie in einem oder mehreren der Ansprüche 2 bis 15 definiert sind.
- 30 18. Zelle und/oder Nachkommenschaft davon nach einem der Ansprüche 1 bis 17, worin die Zelle und/oder die Nachkommenschaft den in Anspruch 1 definierten Vektor enthält, als Medikament.
- 35 19. Zelle und/oder Nachkommenschaft davon nach Anspruch 18 als Medikament, das Zellen Resistenz gegen eine Infektion durch Retroviren verleiht.
- 40 20. Zelle und/oder Nachkommenschaft davon nach Anspruch 18 als Medikament, das Zellen Resistenz gegen eine Infektion durch Retroviren verleiht, die gewählt sind aus der Gruppe, die besteht aus Human-T-Zellen-Lymphotroph-Virus, Human-Immunodefizienz-Virus, Lymphadenopathie-Virus, Leukämie-Virus, Sarkom-Virus und Virus, das lymphotrophe Krankheiten verursacht.
- 45 21. Zelle und/oder Nachkommenschaft davon nach Anspruch 18 als Medikament, das Zellen Resistenz gegen eine Infektion durch HIV verleiht.
- 50 **Revendications**
1. Cellule modifiée en ayant une résistance à l'infection par un rétrovirus, ladite modification comprenant le fait que ladite cellule contient un vecteur comprenant un polynucléotide qui est transcrit en ARN dans ladite cellule, ledit ARN étant sélectionné à partir du groupe se composant d'ARN complémentaire d'une séquence d'acide nucléique dans

- au moins une région dans le génome dudit rétrovirus, où ladite région est sélectionnée à partir du groupe se composant de la région LTR, de la région U5, de la région U3, de la région 3'R et de la région de codon initial AUG, inhibant ainsi, dans le processus d'infection, au moins un processus sélectionné parmi le groupe se composant de réplication rétro-virale, de transcription inverse et de traduction.
2. Cellule selon la revendication 1, où ledit rétrovirus est un virus sélectionné parmi le groupe se composant d'un virus lymphotrope humain à cellules T, d'un virus d'immunodéficiency humaine, d'un virus lymphadénopathique, d'un virus de leucémie, d'un virus de sarcome et d'un virus provoquant une maladie lymphotrope.
3. Cellule selon la revendication 1 ou 2, où ledit rétrovirus est un virus sélectionné parmi le groupe se composant du virus HIV, du virus de leucémie féline, du virus HTLV-I, du virus HTLV-II, du virus de leucémie murine et du virus de leucémie aviaire.
4. Cellule selon l'une quelconque des revendications 1 à 3, où ledit polynucléotide est un polynucléotide synthétique ou bien ledit polynucléotide est de l'ADN.
5. Cellule selon l'une quelconque des revendications 1 à 4, où ledit vecteur est sélectionné à partir du groupe se composant d'un vecteur viral, d'un vecteur rétroviral et d'un plasmide.
6. Cellule selon la revendication 5, où ledit vecteur est de préférence un plasmide.
7. Cellule selon la revendication 6, où ledit plasmide est sélectionné parmi le groupe se composant de pUC19, de pRSVcat, de pSV2gpt, de pSV2neo, de pRSVneo et de pRSVgpt.
8. Cellule selon l'une quelconque des revendications 1 à 7, où ledit vecteur comprend en outre un premier promoteur qui contrôle la transcription dudit ARN dans ladite cellule.
9. Cellule selon l'une quelconque des revendications 1 à 8, où ledit vecteur comprend en outre un premier terminateur qui contrôle la fin de la transcription dudit ARN dans ladite cellule.
10. Cellule selon l'une quelconque des revendications 1 à 9, où ledit vecteur comprend en outre un marqueur pour la sélection de cellules transformées.
11. Cellule selon l'une quelconque des revendications 1 à 10, où ledit polynucléotide est un polynucléotide dirigeant la transcription d'un seul ARN qui est complémentaire de sites d'hybridation multiples dans le génome du rétrovirus.
12. Cellule selon l'une quelconque des revendications 1 à 11, où ledit polynucléotide est un polynucléotide comprenant en outre un second promoteur qui contrôle la transcription dudit ARN dans ladite cellule.
13. Cellule selon la revendication 12, où ledit promoteur est un promoteur de polymérase III de l'ARN.
14. Cellule selon l'une quelconque des revendications 1 à 13, où ledit polynucléotide est un polynucléotide comprenant en outre un second terminateur qui contrôle la fin de la transcription dudit ARN dans ladite cellule-hôte.
15. Cellule selon la revendication 14, où ledit second terminateur est une séquence de terminateur de polymérase III de l'ARN.
16. Génération des cellules selon l'une quelconque des revendications 1 à 15, contenant le vecteur défini dans la revendication 1.
17. Génération des cellules selon la revendication 16, où ledit polynucléotide, ledit vecteur et ladite infection rétrovirale sont définis comme dans l'une quelconque ou plusieurs des revendications 2 à 15.
18. Cellule et/ou toute génération de cellules selon l'une quelconque des revendications 1 à 17, où ladite cellule et/ou ladite génération contient le vecteur défini dans la revendication 1, utilisée comme un médicament.
19. Cellule et/ou toute génération de cellules selon la revendication 18, utilisée comme un médicament donnant de la résistance aux cellules contre une infection rervorale.
20. Cellule et/ou toute génération de cellules selon la revendication 18, utilisée comme un médicament donnant de la résistance aux cellules contre une infection par des retrovirus sélectionnés parmi le groupe se composant d'un virus lymphotrope humain à cellules T, d'un virus d'immunodéficiency humaine, d'un virus lymphadénopathique, d'un virus de leucémie, d'un virus de sarcome et d'un virus provoquant une maladie lymphotrope.
21. Cellule et/ou génération de cellules selon la revendication 18, utilisée comme un médicament donnant de la résistance aux cellules contre une infection d'un virus HIV.



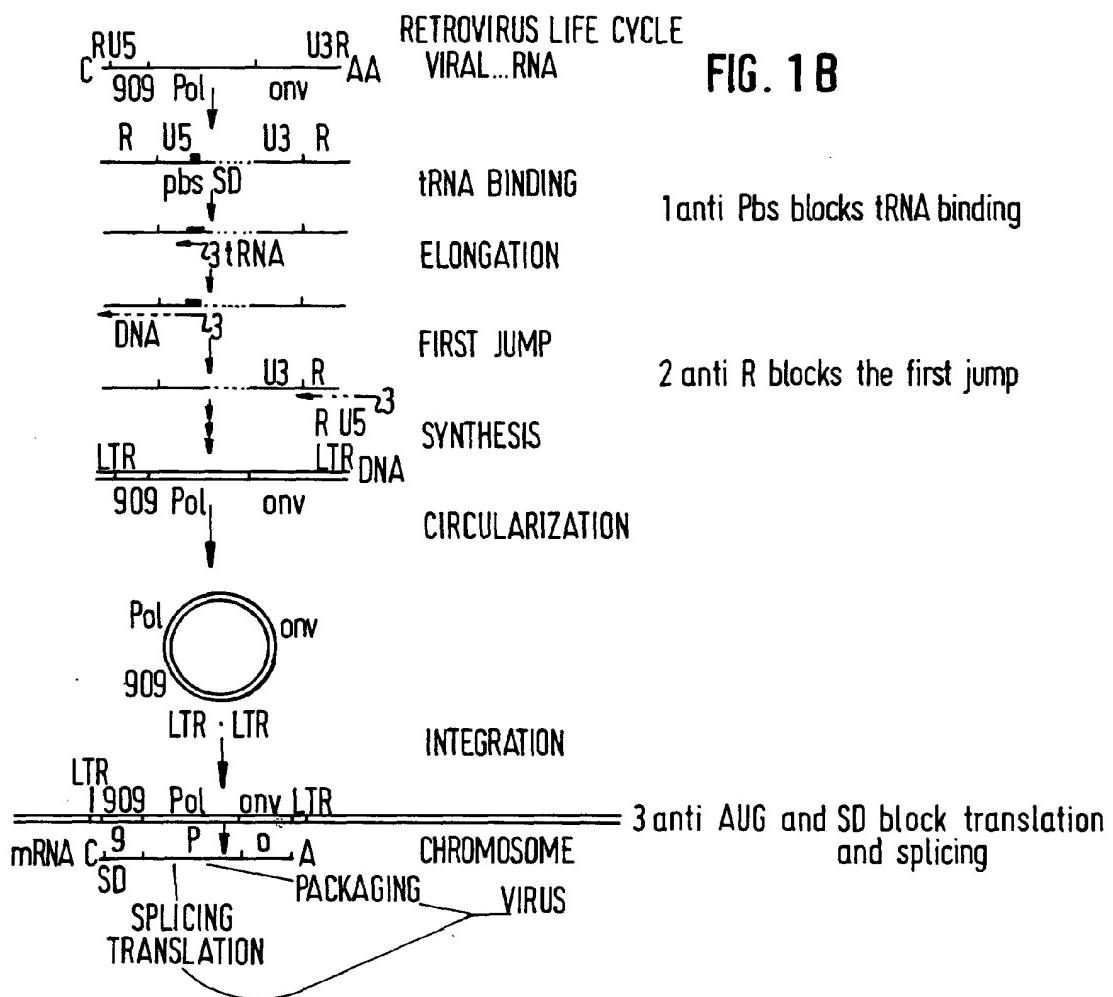


FIG. 1B

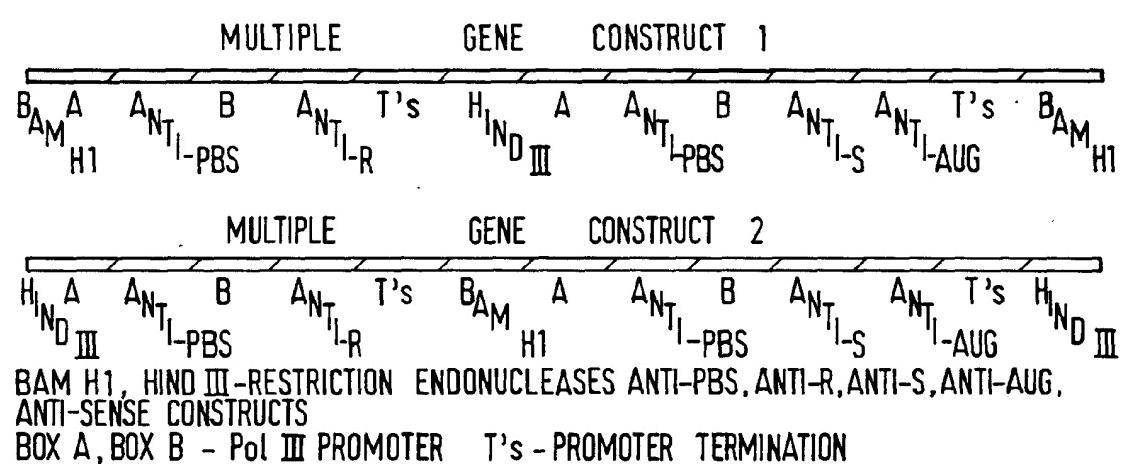


Fig. 2 HIV LTR GENOM

GGUCUCUCUGGUUAGACCAGAUCUGAGGCCUGGGAGCUCUCUGGCUAACUAGGAAACCCACUGCUUA

AGCCUCAAUAAGCUUGCCUUGAGUGCUCAAGUAGUGUGCCGUCUGUUGUGUGACUCUGGUAA

ACUAGAGAUCCCUCAGACCCUUUUAGUCAGUGUGGAAAUCUCUAGCAGUGGCCCGAACAGGGA

CCUGAAAGCGAAAGGGAAACCAAGAGCUCUCUGACGCAGGACUCGGCUUGCUGAAGCGCGCACGGC

AAGAGGCGAGGGCGGCGACUGGUGAGUACGCCAAAAUUUGACUAGCGGAGGCUAGAAGGAGAG

270

MetGlyAlaArgAlaSerValLeuSerGlyGlyGluLeuAspArgTrpGluLysIleArgLeu
AGAUGGGUGCGAGAGCGUCAGUAUUAAGCGGGGAGAAUAGAUCGAUGGGAAAAAUUCGGUUA

ArgProGlyGlyLysLysTyrLysLeuLysHisIleValTrpAlaSerArgGluLeuGluArg
AGGCCAGGGGGAAAGAAAAAUAAAACAUAUAGUAUGGGCAAGCAGGGAGCUAGAACGA

PheAlaValAsnProGlyLeuLeuGluThrSerGluGlyCysArgGlnIleLeuGlyGlnLeuGln
UCCGCAGUUAAUCCUGGCCUGUUAGAAACAUCAGAAGGCUGUAGACAAUACUGGGACAGCUACAA

ProSerLeuGlnThrGlySerGluGluLeuArgSerLeuTyrAsnThrValAlaThrLeuTyrCys
CCAUCUUUCAGACAGGAUCAGAACUUAGAUCAUUAAUACAGUAGCAACCCUCUAUUGU

ValHisGlnArgIleGluIleLysAspThrLysGluAlaLeuAspLysIleGluGluGlnAsn
GUGCAUCAAAGGAUAGAGAUAAAAGACACCAAGGAAGCUUUAGACAAGAUAGAGGAAGAGCAAAAC

LysSerLysLysLysAlaGlnGlnAlaAlaAlaAspThrGlyHisSerSerGlnValSerGln
AAAAGUAAGAAAAAGCACAGCAAGCAGCAGCUGACACAGGACACAGCAGUCAGGUACAGCC

Fig. 3 HTLV-1 LTR GENOM

GGCUCGCAUCUCUCCUCCACGCGCCGCCACCUACCUGAGGCCUCCAUCGCAGCCGAUUGAGUCG

10 20 30 40 50 60

CGUUCUGCCGCCUCCCGCCUGUGGUCCUGAACUGCGUCCGCGUCUAGGUAGUUAAAGCU

70 80 90 100 110 120 130

CAGGUCGAGACCGGGCCUUUGUCCGGCGCUCCUUGGAGCCUACCUAGACUCAGCCGGCUCUCCAC

140 150 160 170 180 190

GCUUUGCCUGACCCUGCUUGCUCAACUCUACGUCUUUGUUUCGUUUUGUGUUCUGCGCCGUUACAG

200 210 220 230 240 250 260

AUCGAAAGUUCCACCCUUUCCUUUCAUUCACGACUGACUGCCGGCUUGGCCACGGCCAAGUAC

270

CGGCGACUCCGUUGGCUCGGAGCCAGCGACAGCCAUCCUAAGCACUCUCAGGAGAGAAUUUAG

360

B

GlyGlnIle

UACACAGUUGGGGGCUCGUCCGGAUACGAGCGCCCCUUUAUCCUAGGCAAUGGCCAAUCCU

PheSerArgSerAlaSerProIleProArgProProArgGlyLeuAlaAlaHisHisTrpLeuAsn

UUCCCGUAGCGCUAGCCC AUUCCCGGACCGCCCCGGGGCUGGCCGUCAUCACUGGUUAAC

PheLeuGlnAlaAlaTyrArgLeuGluProGlyProSerSerTyrAspPheHisGlnLeuLysLys

UUCCUCCAGGCGGCAUAUCGCCUAGAACCCGGUCCUCCAGUUACGAUUUCCACCAGUUAAAAAA

PheLeuLysIleAlaLeuGluThrProAlaArgIleCysProIleAsnTyrSerLeuLeuAlaSer

UUUCUUAAAAUAGCUUUAGAACACCGGCUCGGAUCUGUCCCAUUAACUACUCCCUCCUAGCCAGC

630

LeuLeuProLysGlyTyrProGlyArgValAsnGluIleLeuHisIleLeuIleGlnThrGln

CUACUCCAAAAGGAUACCCGGCCGGUGAAUGAAUUUACACAUACUCAUCCAAACCCA

720

Fig. 4 FeLV LTR GENOM

MLV CCCTGTGCCTT ATTTCAACTAACCAATC A G T TCGCTTCTCGCTTCT
 FeLV AATTCAACCTTCCGTCTCATTTAAACTAACCAATCCCCACGCGTCTCGCTTCT

GTTCGCGCGCTTCCGTCCCCGAGCTCAATAAAA GAGCCCACAACCCCTCACTCGGCG
GTACGCGCGCTT TCT GCT ATAAAAAACGAGCCATCAGCCCC CACA GGCG

CGCCAGTCCTCCGATTGACTGAGTCCCCCGCGTACCCGTATCCAATAAACCCCTTTGC
CGCAAGTCTTGTTGAGACTTCACCCCCCGCGTACCCGTGTA CGAATAAGCCTCTTGC

AGTT GCATCCGACTTGTGGTCTCGGTGTTCTGGG A GGGTCTCCTCT GAGTGA
TGTTTGCATCTGACTCGTGGTCTCGGTCTCCGTGGCACGGGGTCTCATCGCGGAG GA

TTGAC TACCCGTCAGCGGGGGTCTTCATTTGGGGCTCGTCCGGGATCGGAGACCC
A GACCTAC TC CGGGGGTCTTCATTTGGGGCTCGTCCGGGAT A GAGACCC

TG CCCAGGGACCACCGACCCACCACCGGGAGGTAAGCTGGCCAGCAAC TTATCTGTG
CAACCCCAGGGACCACCGACCCACCATCAGGAGGTAAGCTGGCCGGCGACCATATCTGT

TCTGTCCGATTGTCT AGTGTCTATG ACT GATTTTATGCGCCTGCGTCGGTA
TGTCC TTGTGTAAGTGTCTGTCAACTGATCTGATTTT

LeuThrSerSerValSerGlyGly ProValValGluLeuThrSerSerGlu
 CTAGTTAGCTAACTAGCTCTGTATCTGGCGGA CCCGTGGTGGAACTGACGAGTTCGGA
GGCGGTGGAACCGAAGGAGCTGACGAGCTCGTAC

Fig. 4 FeLV LTR GENOM (Contd.)

His Pro Ala Ala Thr Leu Gly Asp Val Pro Gly Thr Ser Gly Ala Val Phe
CACCCGGCCGCAACCCTGGGAGACGTCCA GGG ACT TCGGGGGCCGTTTT
TCCGCCCGCAACCCTGGAAAGACGTTCCACGGGTGTCTGATGTCTGGAGCC TCT A
HetSer Gly Ala Ser S

Val Ala Arg Pro Glu Ser Lys Asn Pro Asp Arg Phe Gly Leu Phe G..
GTGGCCCGACCTGAGTCCAAAAATCCCGATCGTTTGGACTCTTG..
GTGGG ACA G CC ATT GGG GCTCAT CTGTTG..
 er Gly Thr Ala Ile Gly Ala His Leu Phe G..

TABLE 1

Anti-HIV Sequence name	Anti-HIV Sequence ¹	HIV target site ²	Modes of Action ³
1. Anti-R	1-97 (minus strand)	3'R-region of viral RNA and of mRNA	Block '1 st jump' of reverse transcription TAT binding and translation of mRNA
2. R homolog	1-97 (plus strand)	3'R-region of minus strand cDNA	Block '1 st jump' of reverse transcription
3. Anti-PBS	170-210 (minus strand)	PBS site of viral RNA and of plus strand cDNA	Block initiation of reverse transcription and '2 nd jump'
4. PBS homolog	182-199 (plus strand)	3'PBS region of minus strand cDNA	Block '2 nd jump' of reverse transcription
5. False primer	Lys tRNA (with 3' 18 bp substitution)	Any new PBS site of viral RNA	Initiate reverse transcription at improper site
6. False template	PBS homolog (with 5' false tail)	Primer complex and secondary site	"Disarm" primers, produce anti-viral cDNA
7. Polypurine homolog	8630-8670 (plus strand)	Polypurine complement in minus strand cDNA	Block proper initiation of plus strand DNA synthesis

TABLE 1 (Contd.)

Anti-HIV Sequence name	Anti-HIV Sequence ¹	HIV target site ²	Modes of Action ³
8. Anti-S	270-340 (minus strand)	Acceptor site for 1 st TAT splice and GAG initiation codon of GAG translation of mRNA	Block splicing needed for TAT translation and initiation
9. Anti-TAT-S	5340-5430 (minus strand)	Donor site for 1 st TAT splice and TAT initia- tion codon of mRNA	Block splicing needed for TAT translation and initiation of TAT translation
10. Anti-TAT-S	5610-5640 (minus strand)	Acceptor site for 2 nd TAT splice of mRNA	Block mRNA splicing needed for TAT trans- lation
11. Anti-TAT-S	7940-7970 (minus strand)	Donor site for 2 nd TAT splice of mRNA	Block of mRNA spli- cing needed for TAT translation
12. TAT repressor	5530-5593 (plus strand)	5'end of mRNA	Block binding of TAT activator
13. ART repressor	7956-8080 (plus strand)	ART binding site of mRNA	Block binding of ART activator

Fig. 6 HTLV-1 Anti-R Gene Construct

GAAAC	AAAGA	CGTAG	AGTTG	AGCAA	ACAGG	COM
CTTTG	TTTCT	GCATC	TCAAC	TCGTT	CGTCC	REV

Anti-CAP Gene Construct

GTGAA	GGAGA	GATGC	GAGCC		COM
CACTT	CCTCT	CTACG	CTCGG		REV

Fig. 7 FeLV Anti-R Gene Construct

GATGC	AAACA	GCAAG	AGGCT	TTATT	CGTAC	ACGGG	COM
CTACG	TTTGT	CGTTC	TCCGA	AATAA	GCATG	TGCC	REV
TACCC	GGCG	GTCAA	GTCTC	AACAA	AGACT	TGCGC	COM
ATGGG	CCCGC	CAGTT	CAGAG	TTGTT	TCTGA	ACGCG	REV

Fig. 8 HIV Anti-R Gene Construct

ACTTG	AAGCA	CTCAA	GGCAA	GCTTT	ATTGA	GGCTT	COM
TGAAC	TTCGT	GAGTT	CCGTT	CGAAA	TAACT	CCGAA	REV
AAGCA	GAGGG	TTCCC	TAGTT	AGCCA	GAGAG	CTCCC	COM
TTCGT	CACCC	AAGGG	ATCAA	TCGGT	CTCTC	GAGGG	REV
AGGCT	CAGAT	CTGGT	CTAAC	CAGAG	AGACC		COM
TCCGA	GTCTA	GACCA	GATTC	GTCTC	TCTGG		REV

Fig. 9 FeLV Anti-PBS Gene Construct

GGGTC	TCTAT	CCCGG	ACGAG	CCCCC	AAATC	COM
CCCAG	AGATA	GGGCC	TGCTC	GGGGG	TTTAG	REV

Fig. 10 HTLV-1 Anti-PBS Gene Construct

GCGCT	CGTAT	CCCGG	ACGAG	CCCCC	AACTG	COM
CGCGA	GCATA	GGGCC	TGTCT	GGGGG	TTGAC	REV

Fig. 11 HIV Anti-PBS Gene Construct

5'	GTCCC	TGTTTC	GGGCG	CCACT	GCTAG	3'
3'	CAGGG	ACAAG	CCCGC	GGTGA	CGATC	5'

Fig. 12 FeLV Anti-AUG Gene Construct

5'	CTAGA	GGCTC	CAGAC	ATCAG	ACACC	CGTGG	3'
3'	GATCT	CCGAG	GTCTG	TAGTC	TGTGG	GCACC	5'

FeLV Anti-S Gene Construct

5'	TCGCC	GGCCA	GCTTA	CCTCC	TGATG	GTGGG	3'
3'	AGCGG	CCGGT	CGAAT	GGAGG	ACTAC	CACCC	5'

Fig. 13 HTLV-1 Anti-S Gene Construct

5' CTTTA AACCTT ACCTA GACGG CGGAC GCAGT 3'
3' GAAAT TTGAA TGGAT CTGCC GCCTG CGTCA 5'

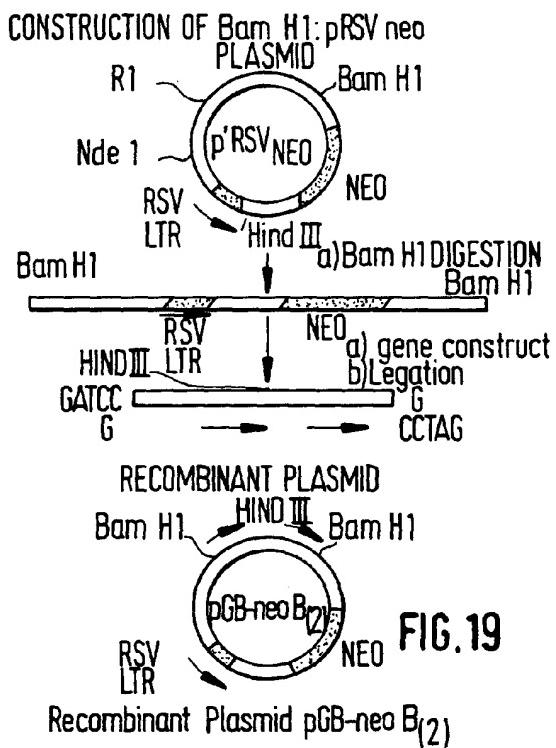
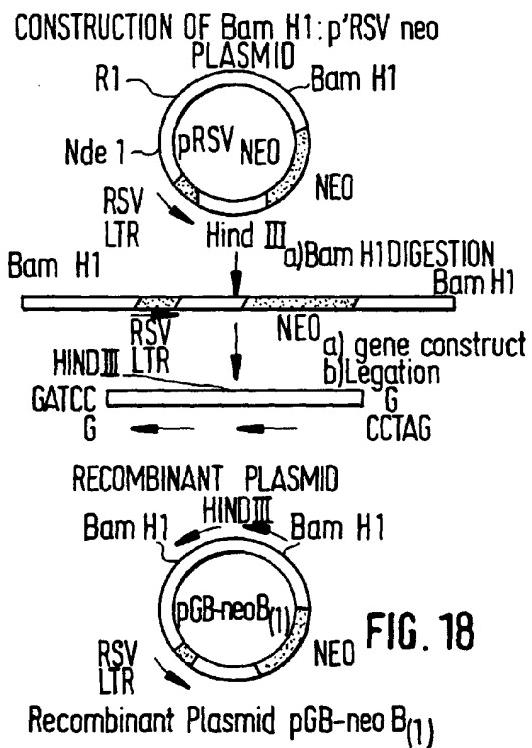
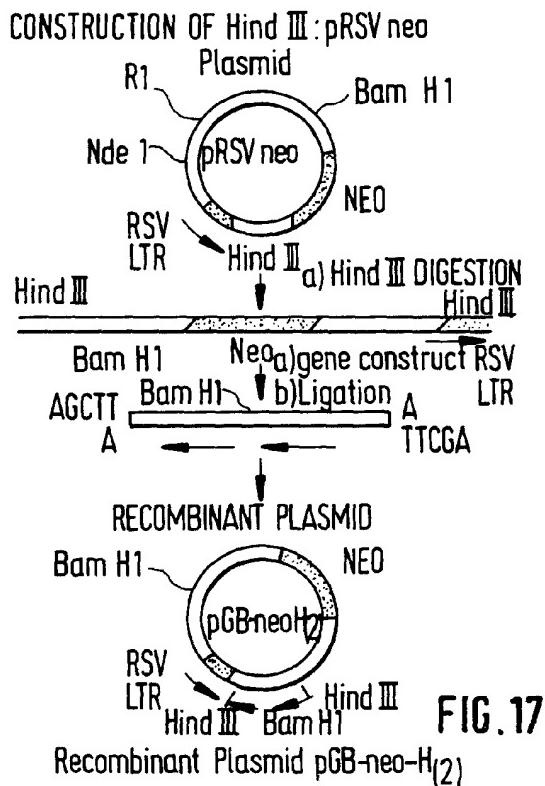
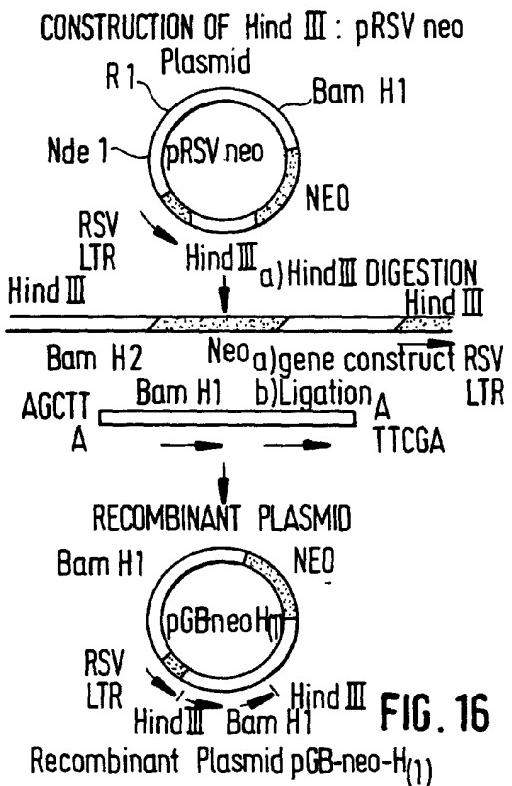
Anti-AUG Gene Construct

5' AGATT GGCCC ATTGC CTAGG GAATA AAGGG 3'
3' TCTAA CCGGG TAACG GATCC CTTAT TTCCC 5'

Fig. 14 HIV Anti-S, Anti-GAG & Anti-AUG Gene Construct

5' TGACG CTCTC GCACC CATCT CTCTC CTTCT AGCCT 3'
3' ACTGC GAGAG CGTGG GTAGA GAGAG GAAGA TCGGA 5'

5' CCGCT AGTCA AAATT TTTGG CGTAC TCACC AGTCG 3'
3' GGCGA TCAGT TTTAA AAACC GCATG AGTGG TCAGC 5'



CONSTRUCTION OF RSV VECTOR FAMILY
FIG. 20

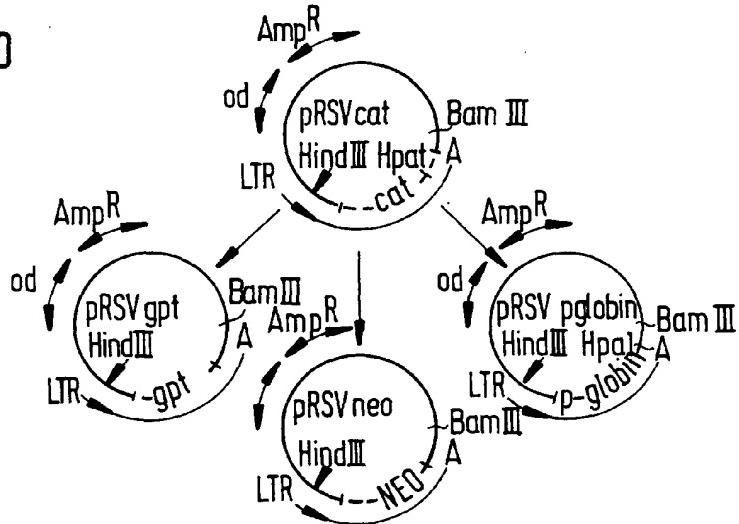


FIG. 21

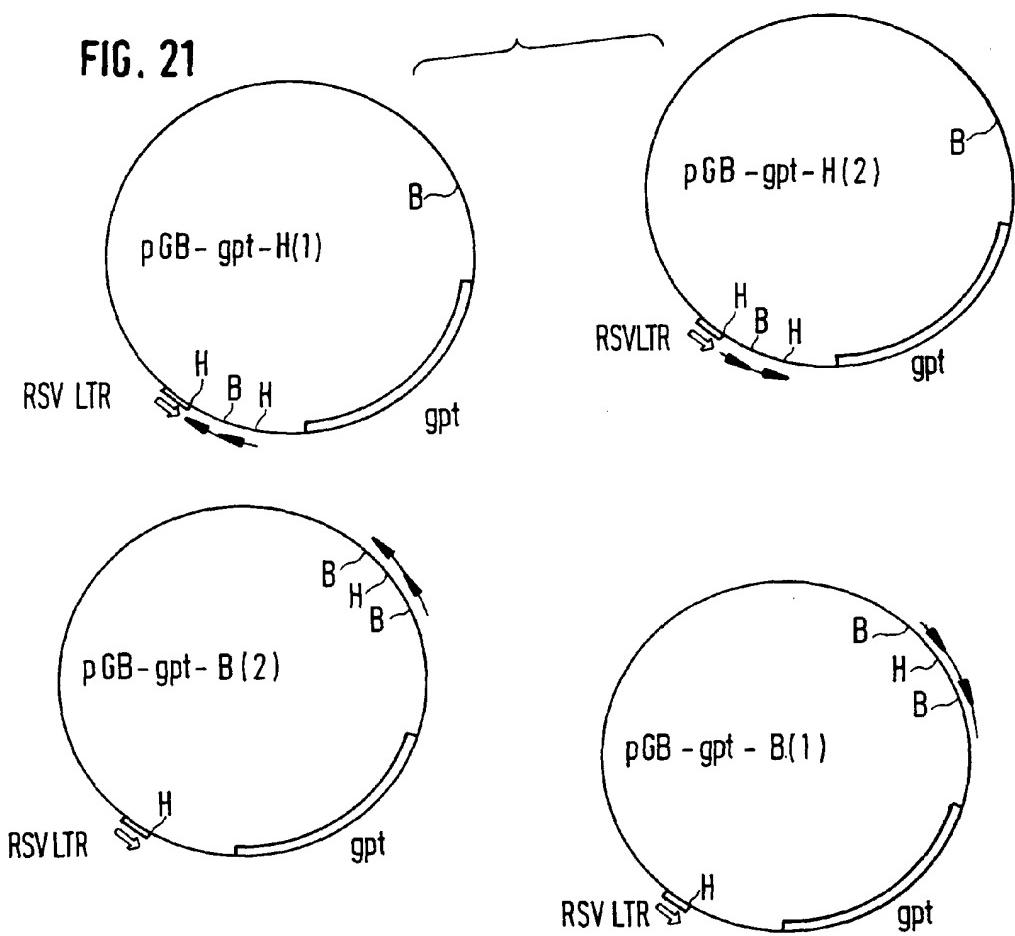


Fig. 22 HIV Gene Construct

Anti-PBS, Anti-AUG, Anti-GAG, Anti-S.D.

GATCC TAGTC AGACA GGCTT TTCAG GTCCC TGTTC GGGCG
----G ATCATC TCTGT CCGAA AAGTC CAGGG ACAAG CCCGC
-BAMHI BOXA.. Anti-PBS

CCACT GCTAG GAGAT CAACT CCAGT TGACG CTCTC GCACC
GGTGA CGATC CTCTA GTTGA GGTCA ACTGC GAGAG CGTGG
.....BOXB.. -----

CATCT CTCTC CTTCT AGCCT CCGCT AGTCA AAATT TTTGG
GTAGA GAGAG GAAGA TCGGA GGCAG TCAGT TTTAA AAACC

CGTAC TCACC AGTCG CCGCC CCTCG TTTTT TTTTT A---
GCATG AGTGG TCAGC GGCAG GGAGC AAAAA AAAAA TTCTGA
----- ----- .STOP. HIND3

Fig. 22 (Contd.)

HIV-Gene Construct

Anti-R, Anti-PBS

AGCTT TGGCA TAGTT GGCTT TTCAG GTCCC TGTTC GGGCG
----A ACCGT ATCAA CCGAA AAGTC CAGGG ACAAG CCCGC
HIND3 BOXA..... -----Anti-PBS -----

CCACT GCTAG GAGTT CGAGA CCAGT ACTTG AAGCA CTCAA
GGTGA CGATC CTCAA GCTCT GGTCA TGAAC TTCGT GAGTT
.....BOXB... -----Anti-

GGCAA TCTTT ATTGA GGCTT AAGCA GTGGG TCCCC TAGTT
CCGTT AGAAA TAACT CCGAA TTCGT CACCC AAGGG ATCAA
R-----Anti-

AGCCA GAGAG CTCCC AGGCT CAGAT CTGGT CTAAC CAGAG
TCGGT CTCTC GAGGG TCCGA GTCTA GACCA GATTG GTCTC
R-----Anti-

AGACC TTTTT TTTTT G----
TCTGG AAAAA AAAAA CCTAG
R---- ...STOP.... BamHI

Fig. 23 FeLV Gene Construct

Anti-R & Anti-PBS

AGGTT TTGGC ATAGT TGGCT GGGTC TCTAT CCCGG ACGAG O
----A AACCG TATCA ACCGA CCCAG AGATA GGGCC TGGTC
Hind3 ----BOXA---- ---- Anti - PBS.....

CCCCC AAATC GGAGT TCGAG ACCAG GATGC AAACA OGCAAG
GGGGG OTTTAG CCTCA AGCTC TGGTC CTACG TTTGT CGTTC O
..... -----BOXB-----.....

AGGCT TTATT CGTAC ACGGG TACCC OGGGCG GTCAA GTCTC
TCCGA AATAA GCATG TGCCC ATGGG CCCGC OCAGTT CAGAG
.... Anti - R...

Bcl1
AACAA AGACT TGATC ATTTT TTTTT G----
TTGTT TCTGA ACTAG TAAAA AAAAA CCTAG
.... ---STOP---- .BamHI

Fig. 23 (Contd.)

FeLV Gene Construct

Anti-PBS, Anti-AUG, Anti-S.D.

GATCC GCAGT CAGAC AGGCA CTATC CCGGA CGAGC OCCCCA
----G CGTCA GTCTG TCCGT GATAG GGCCT GCTCG GGGGT O
BamHI BOX A -----Anti PBS-----

AATGA GAGTT CAACT CCAGT TCGCC OGGCCA GCTTA CCTCC
TTACT CTCAA GTTGA GGTCA AGCGG CCGGT OCGAAT GGAGG
---- BOX B..... ----- SPLICE DONOR SITE--

TGATG GTGGG CTAGA OGGCTC CAGAC ATCAG ACACC CGCGG
ACTAC CACCC GATCT CCGAG OGTCTG TAGTC TGTGG GCGCC
----- AUG SITE.....

TTTTT TTTTT A---
AAAAA AAAAA TTCGA
---STOP----- Hind3

Fig. 24 HTLV-1 Gene Construct

Anti-PBS, S.D. & AUG

1 0	2 0	3 0	4 0				
GATCC	GAGTC	AGACA	GGCTT	TTCAG	GTATC	CCCGG	ACGAG
----G	CTCAG	TCTGT	CCGAA	AAGTC	CATAG	GGGCC	TGCTC
Bam	-----BOXA----			Anti-PBS..		
5 0							
CCCCC	AACTG	GAGGT	CGAGA	CCAGT	CGTAG	AACTT	ACCTA
GGGGG	TTGAC	CTCCA	GCTCT	GGTCA	GCATC	TTGAA	TGGAT
.....	-----BOX B-----			Anti- S.D..		
9 0							
GACGG	CGCAC	GCAGT	AGATT	GGCCC	ATTGC	CCAGG	GAATA
CTGCC	GCGTG	CGTCA	TCTAA	CCGGG	TAACG	GGTCC	CTTAT
.....					-----Anti-AUG-----		
1 3 0							
AAGGG	TTTTT	TTTTT	A----	1 4 0			
TTCCC	AAAAA	AAAAA	TTCGA				
-----STOP... Hind3						

Fig. 24 (Contd.)

HTLV-1 Gene Construct

Anti-R

1 0	2 0	3 0	4 0				
AGCTT	TGGCA	TAGTT	GGCTT	GCGCT	CGTAT	CCC GG	ACGAG
----A	ACCGT	ATCAA	CCGAA	CCCGA	GCATA	GGGCC	TGCTC
Hind3	BOX A...					-----Anti-PBS---
5 0	6 0	7 0	8 0				
CCCCC	AACTG	GAGTT	CGAGA	CCAGT	GTGAA	GGAGA	GATGC
GGGGG	TTGAC	CTCAA	GCTCT	GGTCA	CACTT	CCTCT	CTACG
-----	BOXB...					-----Anti-CAP---
9 0	1 0 0	1 1 0	1 2 0				
GAGCC	CTTTA	AACTT	ACCTA	GACGG	CGGAC	GCAGT	GAAAC
CTCGG	GAAAT	TTGAA	TGGGT	CTGCC	GCCTG	CGTCA	CTTTG
-----	Anti-S.D. Anti-S.D. (contd.)	-----			
1 3 0	1 4 0	1 5 0	1 6 0				
AAAGA	CGTAG	AGTTA	AGCAA	GCAGG	TTTTT	TTTTT	C----
TTTCT	GCATC	TCAAC	TCGTT	CGTCC	AAAAA	AAAAA	GCTAG
---Anti-R/Anti-AUG	-----				STOP	STOP	-Bam

8 SEGMENTS

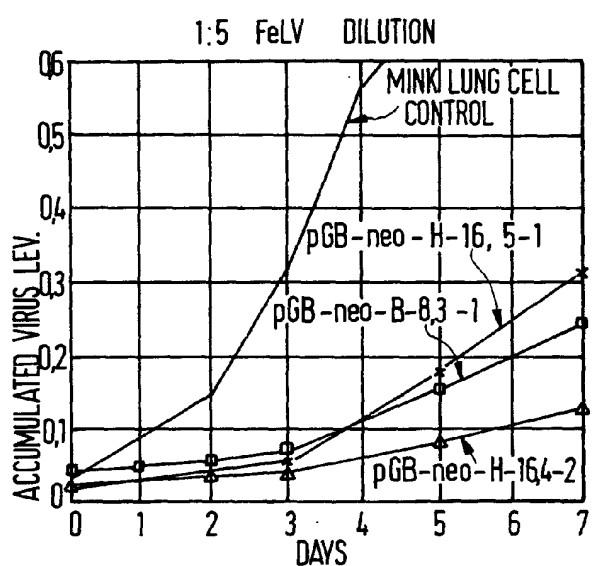


FIG. 25

FeLV DILUTION 1:5

DAYS	CONTROL	pGB-neo B-8 3-1	pGB-neo H-16 4-2 g	pGB-neo H-16 5-1 g
7	-	0,25	0,13	0,31
6	-	0,20	0,11	0,25
5	-	0,16	0,085	0,18
4	0,56	0,12	0,06	0,13
3	0,33	0,06	0,135	0,05
2	0,16	0,055	0,032	0,04
1	0,085	0,05	0,03	0,03
0	0,03	0,04	0,03	0,02

FIG. 26

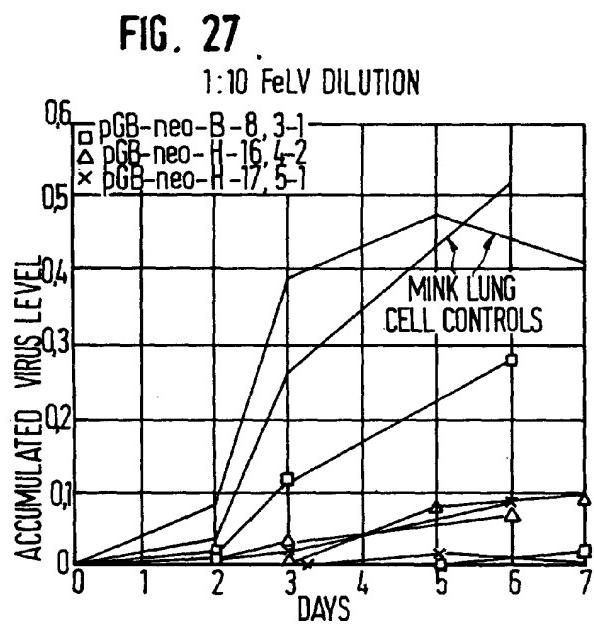


FIG. 27

FeLV DILUTION 1:10

DAYS	CONTROLS	pGB-neo B-8 3-1	pGB-neo H-16 4-2 g	pGB-neo H-16 5-1 g
7	0,41	-	0,015	-
6	0,46	0,51	0,01	0,28
5	0,48	0,435	0	0,23
4	0,44	0,355	0	0,18
3	0,85	0,28	0	0,12
2	0,08	0,03	0	0,015
1	0,04	0,015	0	0,01
0	0	0	0	0

FIG. 28

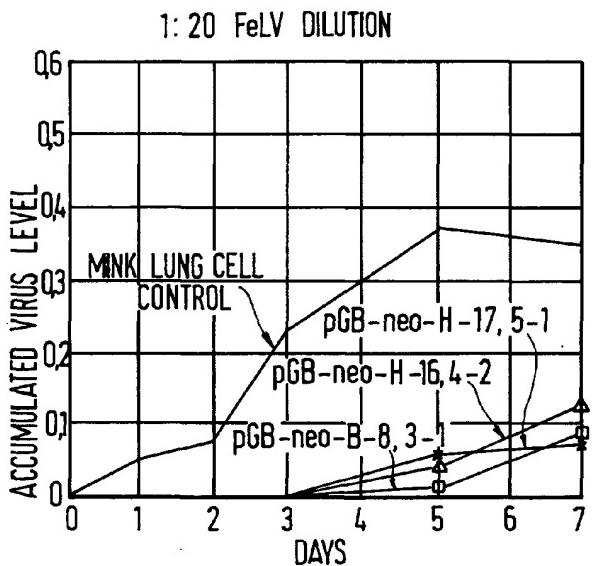


FIG. 29

FeLV DILUTION 1:20

7	-	0,09	0,14	0,08
6	0,36	0,06	0,09	0,07
5	0,37	0,01	0,05	0,055
4	0,3	0,005	0,025	0,03
3	0,23	0	0	0
2	0,07	0	0	0
1	0,05	0	0	0
0	0	0	0	0

DAYS

CONTROL pGB-neo pGB-neo pGB-neo
B-8 H-16 4-2 g 5-1 g

FIG. 30

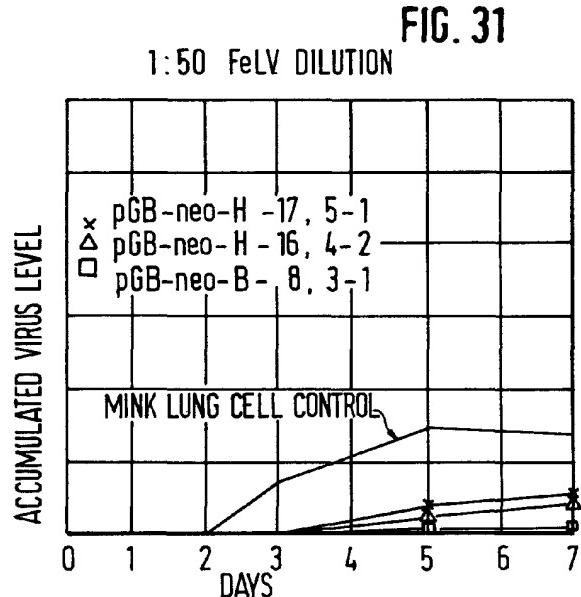


FIG. 31

Fe LV DILUTION 1:50

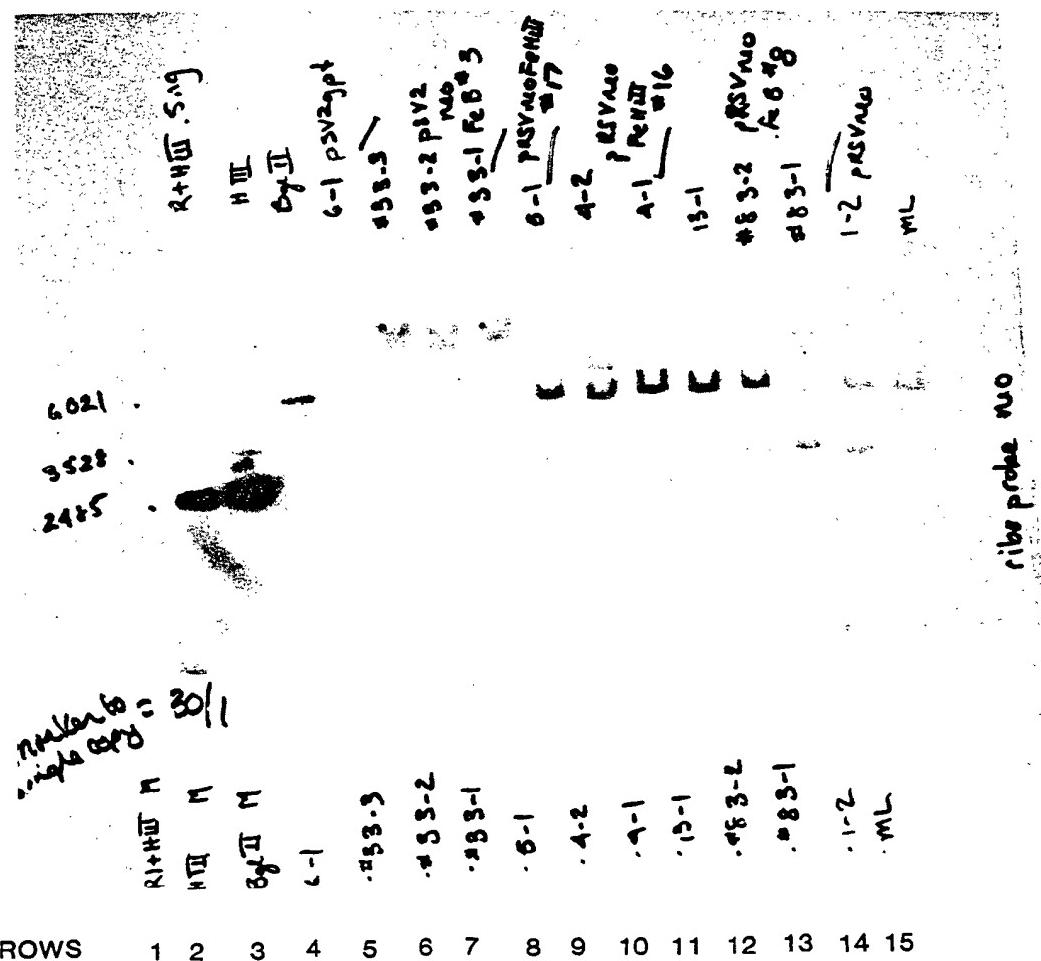
7	-	0,06	0,045	
6	0,14		0,05	0,04
5	0,15		0,04	0,035
4	0,11		0,02	0,015
3	0,07	0	0	0
2	0	0	0	0
1	0	0	0	0
0	0	0	0	0

DAYS

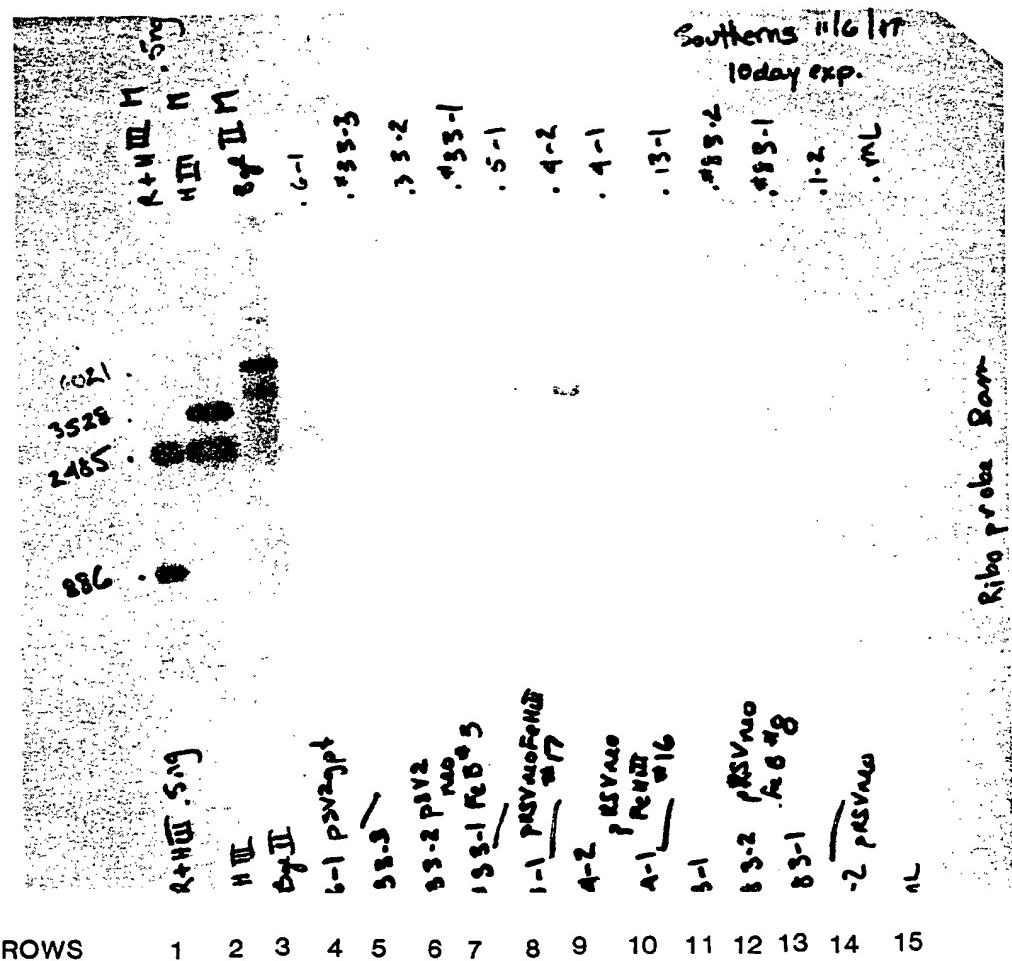
CONTROL pGB-neo pGB-neo pGB-neo
B-8 H-16 4-2 g 5-1 g

FIG. 32

**FIG. 33 SOUTHERN BLOT - HYBRIDIATION
RIBO PROBE Neo**



**FIG. 34 SOUTHERN BLOT - HYBRIDIZATION
RIBO PROBE BAM**



ROWS 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15